REMARKS

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

I. Claim Status

Claims 1, 25, 67 and 81 are currently being amended. Claims 1, 25 and 67 are amended to recited that the particles have a half life of at least 27 days at 40° C. Support for this amendment can be found, *inter alia*, in Table 2. Claim 81 is amended to correct dependency. Claim 84 is added. No new matter is added.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

After amending the claims as set forth above, claims 1-7, 12-32, 37-46 and 67-84 are now pending in this application.

II. Specification

In the Office Action, the Examiner notes that the international publication of the present application, which is the U.S. national phase entry of International Patent Application PCT/GB2003/004202, was not of record. Applicant submits herewith WO 2004/028560 as Exhibit A to make it of record. Applicant notes that this published PCT is the version of the specification available via the USPTO PAIR database.

III. Priority Application

The Examiner alleges that the priority application U.S. Appl. No. 60/414,097 fails to provide support for the claimed invention. Applicant disagrees. Although Example 4 is not included in the parent application in particular, the specification describes the particles as claimed, and appreciates their surprising stability. For example, the paragraph spanning pages 3-4 of the priority application states that the disclosed formulation optimizes stability of

nucleic acid attached to inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent, "thereby promoting shelf-life of the particles and the quality of the nucleic acid delivered to target cells intact." On page 18, lines 7-17, the specification also describes the advantages of polyarginine, describing in particular short homopolymers of $(Arg)_x$, where x is from 2 to 10. Chelating agents as claimed are described beginning on page 19, line 19. While the exact stability, i.e., half life, measurements are not explicitly presented, the property is fundamental and inherent to the disclosed and claimed particles. Therefore, Applicant submit that the priority application, by disclosing the formulation and the increased stability of the claimed particles, supports the present claims.

IV. Claim Rejections under 35 U.S.C. § 112

(i) Indefiniteness

Claims 1-7, 12-32, 37-46 and 67-83 are rejected under 35 U.S.C. 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicant respectfully traverses this rejection.

The Examiner appears to suggest that the phrase "a half life of greater than 20 days at 40° C" is undefined because certain conditions of the formulation are not specifically recited. As noted above, independent claims 1, 25 and 67 now recite that the particles have a half life of at least 27 days at 40° C. Thus, all conditions have been defined such that one of skill in the art would readily recognize the metes and bounds of the claims. Examples 1 and 2 provide ample guidance on how to make the particles, as well as how to measure the half life of the nucleic acid, such as by gel electrophoresis or HPLC meethods.

Applicant respectfully disagrees with the Examiner that conditions, such as pressure, exposures to extraneous components such as acids, bases and enzymes, or length of the DNA, must be defined for one to understand what the phrase "a half life of at least 27 days at 40° C" means in the claims. Acceptability of claim language depends on whether one of ordinary skill in the art would understand what is claimed, in light of the specification. *See, e.g.,*

M.P.E.P. § 2173.05(b). The claims recite the elements required to make the inventive particles. The term "half life" is well known in the art, as shown by the definition attached as Exhibit B. Specifically, this definition recites "[t]he time of survival of half the individual members of an unstable system." While this definition is from a scientific compendium published in 1983, this well-known term has not substantially changed to this day. Therefore, one of skill in the art, upon reading the specification, would have been able to make the inventive particles, as well as identify particles that fall within the scope of the claims, regardless of additional unrecited components mentioned by the Examiner.

Contrary to the Examiner's assertion, those skilled in the art would have easily understood the meaning of the claim phrase in question, which recites well-known terms and easily measured parameters. As such, a skilled artisan would have had no difficulty in ascertaining whether particles infringed Applicant's claims or not.

The dependency of claim 81 has been corrected to recite dependency to claim 80.

As the claims have clear metes and bounds, Applicant respectfully request that the rejection be withdrawn.

(ii) New matter

Claims 1-7-12-32, 37-46 and 67-83 are rejected under 35 U.S.C. 112, first paragraph, as allegedly failing to comply with the written description requirement for comprising new matter. Applicant respectfully traverses this rejection.

As stated in M.P.E.P. § 2163.07, "[a]mendments to an application which are supported in the original description are NOT new matter." Applicant respectfully asserts that the present amendment is supported in the as-filed specification.

The Examiner claims that the application lacks "evidence that the Applicant conveyed to the Artisan, either implicit or explicit, that the invention was limited [to] the broad generic particles claimed." Office Action, page 6. Further, the Examiner states that the data in the specification, specifically the data provided in Tables 2 and 5, are a "hodge-podge" of

stabilities used in an "obviousness-type" argument to overcome the art, and thus do not show possession of the invention. Applicant strongly disagrees.

First, the application as filed states that stability is a notable characteristic of the claimed particles. For example, paragraph [0010] of the published application, U.S. Pat. Appl. Publ. No. 2006/0153804, states "[a] new formulation has been developed to optimize both stability of nucleic acid attached to carrier particles, thereby promoting shelf-life [i.e., increasing the half life] of the particles and the quantity of nucleic acid delivered to target cells intact . . ." The use of condensing agents to enhance stability is discussed generally in paragraph [0065] and arginine homopolymers more specifically in paragraphs [0071]-[0072]. The experiments described in the Examples are specifically designed to test stability, as measured, for example, in terms of half life at a certain temperature. Therefore, the claimed invention, particles with comprising metal carrier particles deposited with a nucleic acid in the presence of a homopolymer of arginine and a metal ion chelator with enhanced stability, i.e., half life, is described, both in general principles and specifically in the Examples. Numerous examples of the claimed particles have actually been reduced to practice and shown to have enhanced stability. Addition of the half life limitation merely makes explicit that which is implicit in, and affirmatively demonstrated for, the inventive particles.

The examples are replete with comparative data testing the half life of claimed particles. Far from a "hodge podge" of data, the examples provide data from numerous formulations that show increased stability, i.e., greater half life, of the claimed particles as compared to those described in the prior art (e.g., Sanford and Oard, teaching the use of spermidine $CaCl_2$ rather than a homopolymer of arginine). At various concentrations and temperatures, the data confirms that the claimed particles reproducibly have the surprising stability as claimed. For example, arginine concentration tested ranges from 0.3 mg/ml (Table 2) and 1.13 mg/ml (Table 4). Likewise, different chelators are tested, all at different temperatures with different saccharides. By making so many comparisons, the data more robustly shows that even under various conditions, the claimed particles have increased stability, i.e., a greater half life than particles obtained in the presence of spermidine $CaCl_2$.

The Examiner points to a single formulation, TA101.4 of Table 2 as not having increased stability as compared to the spermidine $CaCl_2$ controls. This one formulation (out of many) is the only instance in which a recited particle did not exhibit increased stability (TA201.2 of Table 5 does not have a chelator, and thus does not fall within the claims). A single nonoperative embodiment does not render the claims unpatentable under 35 U.S.C. § 112, first paragraph. Indeed, the Patent Office specifically states:

> The presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort than is normally required in the art.

M.P.E.P. § 2164.08(b)(internal citations omitted). The present specification provides extensive working examples for determining the stability of the particles, and at least seventeen formulations with increased stability are described in Tables 2, 4, 5 and 12, as tested *in vitro* and *in vivo* (Table 6). Thus, although one particle formulation encompassed by the present claims may not be suitable for use in the present invention, a person of skill in the art would readily be able to ascertain their suitability using routine methods as provided in the present specification.

Because the instant specification provides ample and literal guidance for the claimed invention, the claims cannot comprise new matter. Therefore, Applicant respectfully requests that the rejection be withdrawn.

V. Claim Rejections under 35 U.S.C. § 103

The Supreme Court has reaffirmed the *Graham* factors for determining obviousness in *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727,1739 (2007) (holding that the proper inquiry for determining obviousness is whether the improvement is more than the predictable use of prior art elements according to their established functions). Further, the Court still requires that the reasoning used to combine the elements in the fashion claimed be made explicit.

(i) Sanford, Balhorn, Oard

Claims 1-5, 7, 12-13, 17-20, 22-30, 32, 37, 38, 42-45 and 67 remain rejected, and claim 73 is newly rejected, under 35 U.S.C. 103(a) as allegedly being unpatentable over U.S. Patent No. 5,204,253 (Sanford) and Balhorn (*Mol. Reprod. Dev.*, (2000) 56: 230-34), as evidenced by Oard (*Plant Cell. Tiss. and Org. Cult.*, (1993) 33(3): 247-50). Applicant respectfully traverses this rejection. One of skill in the art would not have substituted the Balhorn arginine-rich peptides for the spermidine and calcium chloride disclosed in Sanford (or Oard) for use in making particles for ballistic delivery. Those skilled in the art would have had no motivation to combine Sanford and/or Oard with Balhorn in the first instance, and would have had no reasonable expectation of success if they did.

The claimed invention is drawn to particles obtainable by depositing a nucleic acid on inert metal carrier particles in the presence of (i) a homopolymer of arginine of the formula $(Arg)_x$, wherein x is from 2 to 10, or a physiologically acceptable salt thereof; and (ii) a metal ion chelating agent; wherein the particles have a half life of at least 27 days at 40° C. Thus, both an arginine homopolymer and chelator are required during deposition of the nucleic acid on the particle, as the synergistic effect of having both present is critical for achieving the surprising stability found in the claimed particles.

This unexpected synergistic effect on particle stability by the combination of short homopolymers of arginine with a chelator is supported by data showing the absence of a chelator negates the increased stability (TA201.2 of Table 5) as well as data showing even using a longer homopolymer of arginine, approximately 80 monomers, is less effective, as discussed in paragraph [0193]. This improved stability appears to be due to improved attachment of the DNA to the particles, as explained in Applicant's previous Reply. Therefore, short homopolymers of arginine are not merely interchangeable with spermidine (as disclosed in Sanford) or other agents as a stabilizer. Applicant's recited homopolymers of arginine are a surprising improvement on spermidine. Not to mention, adding a chelator provides a more than additive effect for increasing the half life of the particles.

The art cited by the Examiner does not arrive at the claimed invention. Sanford discloses the ballistic delivery of tungsten particles, onto which plasmid DNA is condensed in a buffer containing EDTA in the presence of spermidine/calcium chloride. The preparation of

the particles and their administration takes place on the same day (*see* col. 14, lines 45-46). Therefore, Sanford teaches the use of spermidine, a polyamine that has distinctly different chemical and structural properties from the homopolymers of polyargine used in the present invention, with calcium chloride for DNA condensation onto particles. Furthermore, Sanford fails to address stability of its disclosed particles altogether.

As discussed in Applicant's previous Reply, Balhorn discusses arginine homopolymers as they relate to the decondensation of a DNA-protamine complex, not the long term stability of a metal carrier particle coated with a nucleic acid. Indeed, Balhorn shows dissociation of DNA from an arginine-rich peptide over a matter of seconds. *See* Balhorn, Figure 3 and Table 1. Even with regard to the disclosed toroids, however, Balhorn presents no discussion or suggestion regarding long term stability of DNA-protamine (or DNA-arginine rich peptides), much less the stability of half life measured in days, not seconds, as taught in the present application. As shown in Table 1 of Balhorn, R6 has a decondensation rate of 21.3 seconds. The Examiner fails to explain why one of skill in the art would use a molecule more likely to disassociate from the nucleic acid when developing a more stable formulation. Further, while discussing Balhorn, the Examiner asserts that "with regard to the presence of EDTA on the surface of the particle . . . absent reason to believe otherwise, these particles do have EDTA on their surface." Office Action, page 8. It appears this statement was misplaced, as nowhere in Balhorn is EDTA or any chelator used.

To be thorough, Oard is not specifically addressed in this rejection, but is cited in a later rejection as allegedly teaching the use of gold particles to reduce particle clumping. As an initial matter, Applicant notes that like Sanford, Oard teaches the use of spermidine $CaCl_2$ to condense DNA onto a microparticle. Page 249, 1st col., bottom ¶. Oard, like Sanford, completely fails to suggest the use of a different polymer for this process, but rather states that prior to DNA precipitation, one can rinse microcarriers with poly-L-lysine. *Id,* 1st col., second from bottom ¶. In this context, Oard states "[t]he use of gold flakes and poly-L-lysine did reduce clumping relative to the tungsten particles, but did not eliminate the problem entirely." Page 249, 2nd col., 1st ¶. Thus, this reference suggested that the use of gold flakes and pre-washing with poly-L-lysine might help reduce clumping, but did not teach or suggest the use of poly-L-lysine instead of spermidine $CaCl_2$. Moreover, in light of teachings in other

prior art references (see discussion below regarding Adami, for example), one of skill in the art would have expected that only longer lysine homopolymers might be effective in any event. Finally, like Sanford, Oard did not address stability issues because the disclosed "microcarriers" were prepared and used "as soon as possible after precipitation because the amount of clumping increased over time." Oard, page 249, 2nd col., 1st ¶.

The Examiner asserts that one of skill in the art would be motivated to substitute the arginine rich peptides of Balhorn for the spermidine component of Sanford. However, one of skill in the art would have no expectation of success in mixing and matching disparate elements of two very different techniques. As discussed above, Sanford is drawn to ballistic delivery of tungsten particles coated in plasmid DNA. Balhorn, in contrast, is drawn to DNA-protamine "toroids" for gene delivery to sperm, using the arginine-rich polypeptides to enhance decondensation of the DNA to for quicker uptake of the DNA. One of skill in the art would not include an agent that decondenses (disassociates) more readily, and therefore is more suitable for gentle *in vivo* transfection of sperm, in a ballistic particle delivery system, where the DNA must remain stably attached to the particle though the stress of bombardment.

The Examiner's statement on page 14 of the Office Action that the increased dissociation rate disclosed in Balhorn is unrelated to the off rate of DNA/protein on the microparticle is conclusory and unsupported. If a reagent is important to increased stability of the particle, one of skill in the art would naturally assume that a reagent that actually dissociates more readily from the particle is detrimental to the stability of the particle. Here, Applicant has shown that short homopolymers of arginine are required for the increased stability, despite the teachings of Balhorn showing its tendency to disassociate. Completely unaddressed by the Examiner is that neither Sanford nor Balhorn (nor Oard) provide any expectation that short homopolymers of arginine could even be used to condense DNA onto metal particles at all.

Furthermore, even if the substitution was made, one of skill in the art would not have expected the greater stability of the claimed particles. Indeed, in view of the teachings of Balhorn, one would have actually expected a decrease in stability due to the greater dissociation from DNA displayed by the arginine homopolymers used therein. As explained

previously, this increase in stability using shorter polymers of arginine, as required by the claimed invention, was very surprising, especially in view of what was known in the art at the time of filing the present application, such as evidenced by Adami et al., *J. Pharm. Sci.*, 87:678-683 (1998). According to Applicant's filing records, Applicant submitted this article with their last response. Nonetheless, Applicant provides this reference again herewith as Exhibit C for the Examiner's consideration.

Adami taught, for example, that a polymer of 18 lysine monomers protected DNA from both enzymatic and sonicative-induced degradation, while a shorter polymer of 8 lysine failed to protect DNA from degradation when used as a DNA condensation agent. *See, e.g.*, Abstract and first full paragraph of page 682, left column. Like arginine, lysine is also a basic amino acid with similar chemical structure and properties. Thus, one of skill in the art would have likely drawn conclusions for arginine on the basis of lysine data due to their similar chemical nature, more so than other compounds, such as spermidine.

The Examiner asserts that protection from degradation is not motivation to obviate the claims, but Applicant points out that protection from degradation is the entire point of increased stability. By decreasing degradation, one enhances stability, i.e., increases particle half-life. The invention is drawn to more stable particles, and whether the degradation protected against is enzymatic or physical is irrelevant, particularly since they are experimental tools for testing real world stability. Based on the evidence provided in Adami, one of skill in the art would have expected that <u>longer</u> polymers would be required for enhanced stability, when in fact the present invention has the opposite characteristic.

On page 10 of the Office Action, the Examiner questions whether the invention is drawn to the use of poly-arginine, chelators or both. As discussed above, the invention relies on the surprising synergistic effect of using <u>both</u> short homopolymers of arginine (2-10 monomers) with a chelator. While the Examiner appears to questions the statistical validity of the data provided, Applicant respectfully assert that the actual, working data provided in the Examples are more than sufficient to demonstrate this surprising effect. Rather than routine optimization, where an increase may, at best, be a few percentage points different, the present invention shows at least 30% longer half life (compare 27 days of TA101.2 with

spermidine/$CaCl_2$ in Table 2). In fact, the stability of the inventive particles is usually several times greater than controls (compare TA101.1, TA101.3 with controls in Table 2 or TA201.5 with controls in Table 5). Indeed, the stability of the particles are tested again and again in Tables 2, 5 and 12, with consistent results. Thus, far from mere routine optimization, the present invention provides a surprise improvement.

Finally, the Examiner asserts that because particles are claimed, rather than "the composition after storage for 20 days," then the limitation cannot render the claims nonobvious. Applicant again disagrees. The claims are to particles that have a nucleic acid deposited on them in the presence of a $(Arg)_{2-10}$ and a chelator. These particles have a surprising property in their increased stability, which was made explicit by reciting that they must have a certain stability as measured in half life, a common and widely used method for determining stability of a substance. There is no requirement that the particles must actually be stored for the duration of a half life. Instead, the half life is a convenient measuring technique. Any attempt to require the actual storage of the particles in the claims is arbirtrary and erroneous.

Thus, for at least the foregoing reasons, those skilled in the art would not have considered substituting arginine-rich peptides (much less a homopolymer of $(Arg)_{2-10}$ in particular) used in the sperm transfection techniques of Balhorn for spermidine and $CaCl_2$ when making the particles for ballistic delivery as disclosed in Sanford (and Oard), as suggested by the Examiner. Accordingly, the rejection should be withdrawn.

(ii) Sanford, Balhorn, Oard, Cherng

Claims 1-5, 7, 12-15, 17-30, 32, 37-40, 42-46 and 67 remain rejected, and claims 73, 79, 80, 82 and 93 are newly rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Sanford and Balhorn, as evidenced by Oard, as applied to claims 1-5, 7, 12-13, 17-20, 22-30, 32-38, 42-45, 67 and 72, above, and further in view of Oard and Cherng (*Pharm. Res.*, 16(9): 1417-23 (1999). Applicant respectfully traverses this rejection.

The deficiencies of Sanford, Balhorn and Oard are addressed in detail above and remain applicable for the present rejection. Specifically, one of skill in the art would not have

been motivated to use the arginine-rich fast-disassociation peptides of Balhorn in the ballistic delivery methods of Sanford or Oard, nor would there be any expectation of success in such a combination. Cherng does not overcome these deficiencies, as at best, it teaches the use of sucrose in polymer-plasmid complexes.

Cherng did not use metal particles in its study, instead using a polymer-plasmid polyplex. The polymer used was a methacrylate-based polymer, which is chemically very dissimilar to short homopolymers of arginine. Cherng carefully notes that the results are only applicable to the polyplexes described therein, and at best extended to polyplexes and lipid formulations (page 1423, second paragraph). Thus, one of skill in the art is explicitly cautioned from applying the conclusions made therein to other systems. The Examiner ignores this teaching away, as he asserts "[t]here is no reason to believe it would not apply to other situations." In the face of this explicit cautioning by the authors, however, one of skill in the art are would not have had any reason or motivation to adapt the teachings of Cherng to other systems. The Examiner cites numerous articles in support of his assertion that it was generally known that sugars were known to enhance stability. Such references are even further afield from the present invention than Cherng, as they relate to using sugars in aqueous protein solutions and as a cryoprotectant. None of these references teach or suggest the addition of a sugar to the particles of the present invention.

More importantly, Cherng does not provide any disclosure that would overcome the previously discussed failure of cited combination of references to teach or suggest the surprising stability of the present inventive particles. The effect of short homopolymers of arginine and a chelator on particle stability is not discussed in this reference or the others. As this stability is surprising in view of the art known at the time of filing, as discussed above, the present invention is nonobvious. Therefore, Applicant respectfully requests that the rejection be withdrawn.

(iii) Sanford, Balhorn, Oard, Cherng, Barman, Livesey

Claims 1-7, 12-32, 37-46, 67-73, 76, 79, 80, 82 and 83 remain and/or are newly rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Sanford and Balhorn, as

evidenced by Oard, and Cherng as applied to claims 1-5, 7, 12-15, 17-30, 32-40, 42-46, 67, 73, 79, 80, 82 and 83 above, and further in view of U.S. Publication No. 2004/0142475 (Barman), as further evidenced by U.S. Patent No. 6,194,136 (Livesey). Applicant respectfully traverses this rejection.

The deficiencies of Sanford, Balhorn, Oard and Cherng are addressed in detail above and remain applicable for the present rejection. Specifically, one of skill in the art would not have been motivated to use the arginine-rich fast-disassociation peptides of Balhorn in the ballistic delivery methods of Sanford or Oard, nor would there be any expectation of success in such a combination. Barman and Livesey do not overcome these deficiencies.

Barman is cited by the Examiner as allegedly teaching that saccharides may be used to stabilize nucleic acid protein complexes, and that HPV, HIV, HBV and HSV antigens may be encoded as transgenes for the expression of antigens. Similarly, Livesey is cited as allegedly demonstrating that sugars, including raffinose, can be used as stabilizers. However, as discussed in Applicant's previous response, neither of these references overcome the defect of the other references, which is the failure to teach or suggest the surprising stability of the claimed particles. Barman teaches the use of polymeric microparticles, not inert metal particles, and mentions that stabilizers may be used, with examples including sugars and cationic peptides. *See* paragraphs 41 and 46. Barman neither discloses nor suggests the use of short homopolymers of arginine and a chelator, nor their effect on stability of the particles. Likewise, Livesey provides only general disclosure of cryopreservatives, and does not mention inert metal particles, short homopolymers of arginine or chelators. Thus, the addition of these two references to the other references fails to disclose surprising characteristics of the present invention. Applicant respectfully requests that the rejection be withdrawn.

(iv) Sanford, Balhorn, Oard, Cherng, Barman, Livesey and various

Claims 1-7, 12-32, 37-46 and 67-83 are newly rejected under 35 U.S.C. 103(a) as being unpatentable over Sanford and Balhorn, as evidenced by Oard, Cherng and Barman, as further evidenced by Livesey, as applied to claims 1-7, 12-32, 37-46, 67-73, 76, 79, 80, 82

and 83 above, and further in view of the knowledge of the artisan as evidenced by (i) Ramos et al. (1997) Applied and Environmental Microbiology (e.g., ABSTRACT); (ii) Ericksson et al. (2003) Pharmaceutical Research, 20(9): 1437-43 (e.g., ABSTRACT); (iii) Kaushik et al. (2003) Journal of Biological Chemistry, 278(29): 26485-65 (e.g., ABSTRACT); (iv) Garg et al. (2002) Proceedings of the National Academy of Science, USA, 99(25): 15898-903 (e.g., ABSTRACT); (v) More et al. (1998) Hindustan Antibiotics Bulletin, 40(1-4): 1-4 (ABSTRACT ONLY); (vi) Joshi et al. (2001) AAPS PharmSciTech., 2(4): 25 (ABSTRACT ONLY), (vii) Ruan et al. (2003) European Journal of Biochemistry, 270: 1654-61 (e.g., ABSTRACT), and (viii) Schellman (2003) Biophysical Journal, 85(1): 108-25. Applicant respectfully traverses this rejection.

The deficiencies of previously cited references are addressed in detail above and remain applicable for the present rejection. Specifically, one of skill in the art would not have been motivated to use the arginine-rich fast-disassociation peptides of Balhorn in the ballistic delivery methods of Sanford or Oard, nor would there be any expectation of success in such a combination. The remaining references do not overcome this limitation.

The Examiner adds a lengthy list of various references as allegedly teaching the use of trehalose in the present invention. However, as discussed above, these references are not directed to any technology similar to the claimed particles, much less those having the surprising characteristics of the present invention. Mere recitation of many references do not make up for the fact that none overcome the deficiencies of the main references as previously discussed. These references are merely drawn to various protein solutions and cryopreservation, not particles. Thus, the addition of these references to the other references still fails to disclose surprising characteristics of the present invention. Applicant respectfully requests that the rejection be withdrawn.

CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing or a credit card payment form being unsigned, providing incorrect information resulting in a rejected credit card transaction, or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date Aug. 7, 2009

By

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EXHIBIT A

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WO 2004/028560 A1

(54) Title: NUCLEIC ACID COATED PARTICLES

(57) Abstract: Particles are provided which are suitable for delivery from a particle-mediated delivery device. The particles are obtained by precipitating a nucleic acid on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent. Also described are processes for preparing the particles, and therapeutic methods using the particles including methods of nucleic acid immunisation and gene therapy.

NUCLEIC ACID COATED PARTICLES

Technical Field

The present invention relates to nucleic acid coated particles that are suitable for particle-mediated delivery of nucleic acids by a needleless device. In particular, the invention relates to the use of the particles for *in vivo* and *ex vivo* delivery of nucleic acid molecules to mammalian tissue.

Background

Gene therapy and nucleic acid immunisation are promising approaches for the treatment and prevention of both acquired and inherited diseases. These techniques provide for the transfer of a desired nucleic acid into a subject with subsequent *in vivo* expression. Transfer can be accomplished by transfecting the subject's cells or tissues *ex vivo* and reintroducing the transformed material into the host. Alternatively, the nucleic acid can be administered *in vivo* directly to the recipient. However, the *in vivo* delivery method must allow the nucleic acid to enter the cells of the recipient so that the nucleic acid can be expressed.

A number of methods have been developed for gene delivery in these contexts. Of these, transdermal delivery of nucleic acids provides many advantages over oral or parenteral delivery techniques. In particular, transdermal delivery provides a safe, convenient and noninvasive alternative to traditional administration systems, conveniently avoiding the major problems associated with oral delivery (e.g. variable rates of absorption, gastric degradation and metabolism, hepatic first pass effect, gastrointestinal irritation and/or bitter or unpleasant drug tastes) or parenteral delivery (e.g. needle pain, the risk of introducing infection to treated individuals, the risk of contamination or infection of health care workers caused by accidental needle-sticks and the disposal of used needles).

However, transdermal delivery of nucleic acids also presents a number of inherent problems. Passive delivery through intact skin entails the transport of molecules through a number of structurally different tissues. These may include the

stratum corneum (the major barrier), the viable epidermis, the papillary dermis or the capillary walls in order to gain entry into the blood or lymph system. Transdermal delivery systems must therefore be able to overcome the various resistances presented by each type of tissue.

Therefore, a number of alternatives to passive transdermal delivery have been developed. These alternatives include the use of skin penetration enhancing agents or "permeation enhancers" to increase skin permeability, as well as non-chemical modes such as the use of ionophoresis, electroporation or ultrasound. However, these alternative techniques often give rise to their own side effects such as skin irritation or sensitization.

Recently, particle-mediated techniques suitable for transdermal delivery of nucleic acids have been developed. Particles bearing the nucleic acid of interest are accelerated to high velocity and fired into target tissue using a particle accelerating device. *In vivo*, the particles may be fired directly into recipient cells, avoiding the need for cell uptake of the passenger nucleic acid.

Various particle acceleration devices suitable for particle-mediated delivery are known in the art. Existing devices employ an explosive, electric or gaseous discharge to propel the coated carrier particles towards target cells. The Biolistic® device, for example, delivers DNA-coated microscopic gold beads directly into the cells of the epidermis (Yang *et al* (1990) PNAS USA 87:9568-9572). Particles can also be delivered using a needleless syringe device such as that described in U.S.Patent No.5,630,796 to Bellhouse *et al* ("the PowderJect® needleless syringe device").

Particle-mediated devices are intended to allow safe and easy delivery of nucleic acids. However, the physical characteristics of the particles need to be engineered to meet the demands of needleless administration, in which particles are typically fired at very high velocities. The particles need to have a structural integrity such that they can survive the action of, for example, a gas jet of a syringe or ballistic impact with skin or mucosal tissue. It is also important that the particles have a density that enables the particles to achieve sufficient momentum to penetrate

tissue. For nucleic acid delivery however, the particles should be smaller than cell size so that they can penetrate cell membranes without disrupting the cells. Nucleic acids are themselves susceptible to degradation on storage. Therefore, the nucleic acid needs to be maintained in stable conditions when associated with the particle. However, the association of the nucleic acid with the particle should also allow efficient expression of the nucleic acid after delivery to the target cell. Where the nucleic acid encodes an antigen, the means of particle association should also allow immunogenicity of the antigen in a subject.

According to one technique, particles suitable for particle mediated delivery can be formed by coating nucleic acid molecules onto inert metal carrier particles. The carrier particles are selected from materials having a suitable density and size, such as tungsten or gold. A number of methods are known for coating or precipitating DNA or RNA onto gold or tungsten particles. These methods generally combine a predetermined amount of gold or tungsten with plasmid DNA, $CaCl_2$ and spermidine. The resulting solution is vortexed continually during the coating procedure to ensure uniformity of the reaction mixture. After precipitation of the nucleic acid, the coated particles can be transferred to suitable membranes and allowed to dry prior to use, coated onto surfaces of a sample module or cassette, or loaded into a delivery cassette for use in particular particle-mediated delivery instruments.

Summary of the Invention

A new formulation has been developed to optimise both stability of nucleic acid attached to carrier particles, thereby promoting shelf-life of the particles and the quantity of nucleic acid delivered to target cells intact, and also expression and physiological activity of the nucleic acid upon delivery to the target cells. Specifically, it has been found that nucleic acids can be stably attached to inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent.

The nucleic acid can thus be precipitated onto the carrier particles at neutral

rather than alkaline pH, helping to preserve nucleic acid integrity. Furthermore, the particles can then be washed in aqueous or alcoholic solutions without loss of nucleic acid, facilitating incorporation of stability enhancing agents. Condensation of the nucleic acid, together with chemical action of the chelating agent protect the nucleic acid from both physical damage and chemical damage, for example oxidation by free radicals or digestion by endonucleases. The particles of the invention can be delivered to cells by efficient particle mediated delivery. Despite the stability of the nucleic acid particle association, it has been shown that there is efficient expression of the nucleic acid in the target cells. Furthermore immunopotency of nucleic acids encoding antigens has been demonstrated.

Accordingly, the present invention provides particles suitable for delivery from a particle-mediated delivery device, which particles are obtainable by precipitating a nucleic acid on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent.

The particle formulation can be further improved by using one or more sugars, and/or salt in particle preparation, and/or by treating particles with an antioxidant such as ethanol or vitamin A, C or E.

The invention also provides:

- a process for the preparation of particles suitable for delivery from a particle-mediated delivery device, comprising the steps of:

  (i) precipitating a nucleic acid on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent; and

  (ii) collecting the resultant particles;

- a method of nucleic acid immunisation comprising

  (a) providing particles suitable for delivery from a particle-mediated delivery device which particles are obtainable by precipitating a nucleic acid encoding an antigen on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent; and

  (b) administering an effective amount of the particles to a subject;

a method of gene therapy comprising:

(a) providing particles suitable for delivery from a particle-mediated delivery device which particles are obtainable by a precipitating a nucleic acid encoding a therapeutic polypeptide on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent; and

(b) administering an effective amount of the particles to a subject; and

- particles, suitable for delivery from a particle mediated delivery device, which comprise inert metal carrier particles having on their surface a nucleic acid, a metal ion chelating agent and one or more of:

(i) a nucleic acid condensing agent;

(ii) one or more disaccharide and/or trisaccharide sugars; and

(iii) one or more salts.

The invention also provides a dosage receptacle for a particle mediated delivery device, the receptacle containing particles of the invention, as well as a particle mediated delivery device loaded with particles of the invention.

These and other objects, aspects, embodiments and advantages of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Detailed Description of the Preferred Embodiments

Before describing the present invention in detail, it is to be understood that this invention is not limited to particularly exemplified molecules or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting. In addition, the practice of the present invention will employ, unless otherwise indicated, conventional methods of virology, microbiology, molecular biology, recombinant DNA techniques and immunology all of which are within the ordinary skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, *et al.*, *Molecular Cloning: A*

Laboratory Manual (2nd Edition, 1989); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., 1984); *A Practical Guide to Molecular Cloning* (1984); and *Fundamental Virology*, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the content clearly dictates otherwise.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms "nucleic acid molecule" and "polynucleotide" are used interchangeably herein and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. Non-limiting examples of polynucleotides include a gene, a gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

A polynucleotide is typically composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); and thymine (T) (uracil (U) for thymine (T) when the polynucleotide is RNA). Thus, the term nucleic acid

sequence is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

A nucleic acid sequence which "encodes" a selected antigen is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. For the purposes of the invention, such nucleic acid sequences can include, but are not limited to, cDNA from viral, procaryotic or eucaryotic mRNA, genomic sequences from viral or procaryotic DNA or RNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence.

A "vector" is capable of transferring nucleic acid sequences to target cells (e.g., viral vectors, non-viral vectors, particulate carriers, and liposomes). Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning and expression vehicles. A "plasmid" is a vector in the form of an extrachromosomal genetic element.

A "promoter" is a nucleotide sequence which initiates and regulates transcription of a polynucleotide. Promoters can include inducible promoters (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), repressible promoters (where expression of a polynucleotide sequence operably linked to the promoter is repressed by an analyte, cofactor, regulatory protein, etc.), and constitutive promoters. It is intended that the term "promoter" or "control element" includes full-length promoter regions and functional (*e.g.*, controls transcription or translation) segments of these regions.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter operably linked to a nucleic acid sequence is capable of effecting the expression of that sequence when the proper enzymes are present. The promoter need not be contiguous with the sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the nucleic acid sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Recombinant" is used herein to describe a nucleic acid molecule (polynucleotide) of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide with which it is associated in nature and/or is linked to a polynucleotide other than that to which it is linked in nature. Two nucleic acid sequences which are contained within a single recombinant nucleic acid molecule are "heterologous" relative to each other when they are not normally associated with each other in nature.

A "polypeptide" is used in it broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or other peptidomimetics. The subunits may be linked by peptide bonds or by other bonds, for example ester, ether, etc. As used herein, the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is typically called a polypeptide or a protein.

An "antigen" refers to any agent, generally a macromolecule, which can elicit an immunological response in an individual. The term may be used to refer to an individual macromolecule or to a homogeneous or heterogeneous population of antigenic macromolecules. As used herein, "antigen" is generally used to refer to a protein molecule or portion thereof which contains one or more epitopes. For

purposes of the present invention, antigens can be obtained or derived from any appropriate source. Furthermore, for purposes of the present invention, an "antigen" includes a protein having modifications, such as deletions, additions and substitutions (generally conservative in nature) to the native sequence, so long as the protein maintains sufficient immunogenicity. These modifications may be deliberate, for example through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the antigens.

An "immune response" against an antigen of interest is the development in an individual of a humoral and/or a cellular immune response to that antigen. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells.

The term "nucleic acid immunization" is used herein to refer to the introduction of a nucleic acid molecule encoding one or more selected antigens into a host cell for the *in vivo* expression of the antigen or antigens. The nucleic acid molecule can be introduced directly into the recipient subject by transdermal particle delivery. The molecule alternatively can be introduced *ex vivo* into cells which have been removed from a subject. In this latter case, cells containing the nucleic acid molecule of interest are re-introduced into the subject such that an immune response can be mounted against the antigen encoded by the nucleic acid molecule. The nucleic acid molecules used in such immunization are generally referred to herein as "nucleic acid vaccines."

The term "transdermal" delivery intends intradermal (e.g., into the dermis or epidermis), transdermal (e.g., "percutaneous") and transmucosal administration, i.e., delivery by passage of an agent into or through skin or mucosal tissue. *See, e.g., Transdermal Drug Delivery: Developmental Issues and Research Initiatives*, Hadgraft and Guy (eds.), Marcel Dekker, Inc., (1989); *Controlled Drug Delivery: Fundamentals and Applications*, Robinson and Lee (eds.), Marcel Dekker Inc., (1987); and *Transdermal Delivery of Drugs*, Vols. 1-3, Kydonieus and Berner (eds.), CRC Press, (1987). Thus, the term encompasses delivery from a needleless syringe

as described in U.S. Patent No. 5,630,796, as well as particle-mediated delivery as described in U.S. Patent No. 5,865,796.

By "needleless syringe" is meant an instrument which delivers a particulate composition transdermally without the aid of a conventional needle to pierce the skin. Needleless syringes for use with the present invention are discussed throughout this document.

The terms "individual" and "subject" are used interchangeably herein to refer to any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The terms do not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The methods described herein are intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

B. General Methods

The invention is concerned with particle-mediated delivery of nucleic acids. In particular, the invention provides particles which are suitable for delivery from a particle-mediated delivery device, and which are obtainable by a process which comprises or which in some embodiments consists essentially of depositing a nucleic acid on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent.

The carrier particles are selected from metals which have a suitable density and suitable particle size for intracellular delivery from a particle-mediated delivery device. Preferably, carrier particle density is from about 15 to 25 g/ml for example, from about 15 to 23 g/ml or from about 16 to 20 g/ml. Carrier particles may have diameters of from about 0.5 to 10 μm, for example from about 1 to 5 μm. It is

particularly preferred that the carrier particles have a diameter of from about 0.5 to 3 μm , eg from about 1 to 3 μm or 0.5 to 2 μm.

The metal carrier particles are inert in that they are unreactive within the *ex vivo* cells or the body of the subject to which the particles are to be administered. Typically, gold, tungsten, platinum or iridium carrier particles are used. Gold or tungsten particles are preferred. The gold particles may be colloidal gold particles. Gold particles provide uniformity in size (available from Alpha Chemicals in particle sizes of about 1 to 3 μm, or available from Degussa, South Plainfield, NJ in a range of particle sizes including 0.95 μm) and reduced toxicity. Microcrystalline gold (e.g., gold powder A1570, available from Engelhard Corp., East Newark, NJ) provides a diverse particle size distribution, typically in the range of about 0.1 to 5 μm. However, the irregular surface area of microcrystalline gold provides for highly efficient coating with nucleic acids. Tungsten particles are readily available in average sizes of about 0.5 to 2 μm in diameter.

Typically the nucleic acid molecule comprises therapeutically relevant nucleotide sequence for delivery to a subject. It is preferred that the present particles are suitable for use in nucleic acid immunisation or gene therapy. The nucleic acid may thus comprise a sequence capable of providing immunity, for example an immunogenic sequence that elicits a humoral and/or cellular immune response when delivered to a subject. Alternatively, the nucleic acid may comprise one or more genes encoding a therapeutic polypeptide e.g a protein defective or missing from a target cell genome or a non-native protein having a desired biological or therapeutic effect (e.g., an antiviral fuction). The nucleic acid may comprise sequence that encodes a molecule having an antisense or ribozyme function. For the treatment of genetic disorders, functional genes corresponding to genes known to be deficient in the particular disorder can be administered to a subject. Preferably the nucleic acid is DNA.

Suitable nucleic acids for delivery include those used for the treatment of inflammatory diseases, autoimmune, chronic and infectious diseases, including such disorders as AIDS, cancer, neurological diseases, cardivascular disease,

hypercholestemia; various blood disorders including various anemias, thalassemia and hemophilia; genetic defects such as cystic fibrosis, Gaucher's Disease, adenosine deaminase (ADA) deficiency, emphysema, etc. A number of antisense oligonucleotides (e.g., short oligonucleotides complementary to sequences around the translational initiation site (AUG codon) of an mRNA) that are useful in anitsense therapy for cancer and for viral diseases have been described in the art. See, e.g., Han *et al* (1991) *Proc. Natl. Acad. Sci* USA 88:4313; Uhlmann *et al* (1990) Chem. Rev. 90:543, Helene *et al* (1990) Biochim. *Biophys. Acta.* 1049:99; Agarwal *et al* (1988) Proc. *Natl. Acad. Sci.* USA 85: 7079; and Heikkila *et al* (1987) *Nature* 328:445. A number of ribozymes suitable for use herein have also been described . See, e.g., Chec *et al* (1992) *J. Biol. Chem.* 267: 17479 and U.S. Patent No. 5,225,347 to Goldberg *et al.*

For example, in methods for the treatment of solid tumors, genes encoding toxic peptides (i.e., chemotherapeutic agents such as ricin, diptheria toxin and cobra venom factor), tumor suppressor genes such as p53, genes coding for mRNA sequences which are antisense to transforming oncogenes, antineoplastic peptides such as tumor necrosis factor (TNF) and other cytokines, or transdominant negative mutants of transforming oncogenes, can be delivered for expression at or near the tumor site.

Similarly, nucleic acids coding for polypeptides known to display antiviral and/or antibacterial activity, or stimulate the host's immune system, can also be administered. The nucleic acid may encode one of the various cytokines (or functional fragments thereof), such as the interleukins, interferons and colony stimulating factors. The nucleic acid may encode an antigen for the treatment or prevention of a number of conditions including but not limited to cancer, allergies, toxicity and infection by a pathogen such as, but not limited to, fungus, viruses including Human Papilloma Viruses (HPV), HIV, HSV2/HSV1, influenza virus (types A, B and C), Polio virus, RSV virus, Rhinoviruses, Rotaviruses, Hepatitis A virus, Norwalk Virus Group, Enteroviruses, Astroviruses, Measles virus, Par Influenza virus, Mumps virus, Varicella-Zoster virus, Cytomegalovirus, Epstein-Barr

virus, Adenoviruses, Rubella virus, Human T-cell Lymphoma type I virus (HTLV-I), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus, Pox virus, Marburg and Ebola; bacteria including *M.tuberculosis, Chlamydia, N.gonorrhoeae, Shigella, Salmonella, Vibrio Cholera, Treponema pallidua, Pseudomonas, Bordetella pertussis, Brucella, Franciscella tulorensis, Helicobacter pylori, Leptospria interrogaus, Legionella pnumophila, Yersinia pestis, Streptococcus (types A and B), Pneumococcus, Meningococcus, Hemophilus influenza (type b), Toxoplama gondic, Complybacteriosis, Moraxella catarrhalis, Donovanosis, and Actinomycosis;* fungal pathogens including Candidiasis and Aspergillosis; parasitic pathogens including Taenia, Flukes, Roundworms, Amebiasis, Giardiasis, Cryptosporidium, Schitosoma, Pneumocystis carinii, Trichomoniasis and Trichinosis. The nucleic acid my also be used to provide a suitable immune response against numerous veterinary diseases, such as Foot and Mouth diseases, Coronavirus, *Pasteurella multocida, Helicobacter, Strongylus vulgaris, Actinobacillus pleuropneumonia,* Bovine viral diarrhea virus (BVDV), *Klebsiella pneumoniae, E. Coli, Bordetella pertussis, Bordetella parapertussis and Bordetella brochiseptica.* Thus in one aspect, the particles of the present invention may find use as a vaccine.

The invention will also find use in antisense therapy, e.g., for the delivery of oligonucleotides able to hybridize to specific complementary sequences thereby inhibiting the transcription and/or translation of these sequences. Thus DNA or RNA coding for proteins necessary for the progress of a particular disease can be targeted, thereby disrupting the disease process. Antisense therapy, and numerous oligonucleotides which are capable of binding specifically and predictably to certain nucleic acid target sequences in order to inhibit or modulate the expression of disease-causing genes are known and readily available to the skilled practitioner. Uhlmann *et al* (1990) *Chem Rev.* 90: 543, Neckers *et al* (1992) *Int. Rev. Oncogenesis* 3, 175; Simons *et al* (1992) *Nature* 359, 67; Bayever *et* al (1992) Antisense *Res. Dev.* 2: 109; Whitesell *et al* (1991) Antisense Res. Dev. 1: 343; Cook *et al* (1991) *Anti-cancer Drug Design 6: 585;* Eguchi *et al* (1991) *Ann. Rev. Biochem. 60: 631.* Accordingly, antisense oligonucleotides capable of selectively

binding to target sequences in host cells are provided herein for use in antisense therapeutics.

Typically the nucleic acid is provided as an expression vector. Such expression vectors can be routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for expression of an inserted sequence. Suitable vectors would be apparent to persons skilled in the art. By way of further example in this regard reference is made to Sambrook *et al.* 1989.

A suitable expression vector comprises a polynucleotide for use in the present invention operably linked to a control sequence, typically a promoter, which is capable of providing for the expression of the polynucleotide by a host cell. Preferably the vector is suitable for use in a method of gene therapy or nucleic acid immunisation. The vector may be used *ex vivo*, for example to transform a host cell which is then reintroduced into a subject. Alternatively, the vector may be used *in vivo* for direct delivery to a subject.

The vector is typically a plasmid provided with an origin of replication, a promoter for the expression of the polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistence gene.

Promoters and other expression regulation signals are selected to be compatible with the host cell for which expression is designed. Inducible, repressible or otherwise controllable promoters may be used. For expression in mammalian hosts or mammalian host cells, both eukaryotic and phage control elements may be used.

Suitable mammalian promoters include the metallothionein promoter which can be induced in response to heavy metals such as cadmium and β-actin promoters. Tissue-specific promoters are especially preferred. Suitable viral promoters include for example the Moloney murine leukaemia virus long terminal repeat (MMLV

LTR), the Rous Sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (hCMV) immediate early (IE) promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). All these promoters are readily available in the art.

Typically, transcription termination and polyadenylation sequences will also be present, located 3' to each translation stop codon. Preferably, a sequence for optimisation of initiation of translation located 5' to each coding sequence is also present. Examples of transcription termination/polyadenylation signals include those derived from SV40, as described in Sambrook *et al.*, as well as a bovine growth hormone terminator sequence. In addition enhancer elements may be included to increase expression levels. Examples of suitable enhancers include the SV40 early gene enhancer (Dijkema et al (1985) EMBOJ. 4:761) the enhancer/promoter derived from the long terminal repeat of the Rous Sarcoma Virus (LTR) (Gorman et al (1982) *Proc. Natl. Acad. Sci* USA 79: 6777) and elements derived from human or murine CMV (Boshart et al (1985) Cell 41:521) for example, elements included in the CMV intron A sequence.

The nucleic acid condensing agent for use in the invention interacts with a nucleic acid in such a way as to condense the nucleic acid into a more compact structure. The condensed nucleic acid is typically more stable than the uncondensed form, and usually has a more ordered structure, for example, a toroidal or rodlike shape. Condensing agents are typically basic molecules, which interact electrostatically with the nucleic acid to counteract its negative charge. It has been reported, for example, that 88-90% charge neutralisation is required for efficient condensation to occur (Deng H. and V.A. Bloomfield (1999) Biophys J 77:1556-61). Usually, the condensing agent binds to the nucleic acid with a relatively high affinity.

Any suitable nucleic acid condensing agent can be used including cationic polymers and multivalent cations. In one embodiment the condensing agent is a cationic polymer or a physiologically acceptable salt thereof. Such pharmaceutically acceptable salts include for example, mineral acid salts such as hydrochlorides,

hydrobromides, phosphates, sulphates and the like and salts of organic acids such as acetates, propionates, malonates, benzoates and the like. Typically the salt is a hydrochloride or a sulphate.

Preferably the cationic polymer is a polyamine. Polyamines which may be used include protamines, putrescine, spermidine, spermine, hexadimethrine bromide (Polybrene®) polyarginines and polylysines, or physiologically acceptable salts of the foregoing. The cationic polymer may be a peptide containing a polyamine.

Preferably the polyamine is a cationic polymer of basic amino acids. Typically the basic amino acids are selected from lysine, arginine and histidine. Such polyamino acids are readily available from Sigma-Aldrich.

The polyamino acid may be a homopolymer of a basic amino acid. For example, polyarginine, polylysine or polyhistidine. Alternatively the polyamino acid may be a polymer of one or more basic amino acids, optionally also including one or more non-basic amino acids. Thus the polyamino acid may comprise one or more basic amino acids and optionally one or more other amino acids. Such a copolymer typically comprises a majority of basic amino acids. For example, 50 to 100% of the amino acids in the copolymer may be basic. Preferably 60 to 90% or 70 to 80% are basic. In one embodiment at least 75%, for instance at least 85%, 95%, 98%, or 99% of the amino acids in the copolymer are basic. In general, the basic amino acids comprise one or more of lysine, histidine and arginine. Where the copolymer includes one or more non-basic amino acids, these are preferably not acidic amino acids, such as aspartate or glutamate. The one or more non-basic amino acids may include amino acids with aliphatic or aromatic side chains, for example, threonine, proline, tryptophan, serine or phenylalanine.

The amino acids in any of the above polyamino acids may be L or D amino acids. Preferably, L-amino acids are used.

In a preferred embodiment, the polyamine is a homopolymer of arginine $(Arg)_x$ or lysine $(Lys)_x$. Poly-Larginine or poly-L-lysine are preferred, in particular poly-L-arginine. Typically, the homopolymer has a molecular weight of from about 500 to 15000, for example from 500 to 10000, from 500 to 5000, or from 500 to

1000. In one embodiment, x in the above formula may range from 2 to 100, for example from 2 to 50, from 2 to 30 or 2 to 20.

Small pepide homopolymers are particularly preferred, for example, those having a molecular weight in the range 500 to 1500, such as 500 to 1250, or 700 to 1000. Typically, in a small peptide, x has a value of from 2 to 10, for example from 4 to 8. Homopolymers where x = 4 or 6, for example $(Arg)_4$ or $(Arg)_6$ are especially useful in the present invention.

The condensing agent may be a member of the protamine family of proteins for example, protamine sulphate. Protamines are basic proteins, which occur bound to sperm DNA in place of histones. Sperm nuclei therefore provide an excellent source of protamines e.g. salmine from salmon sperm, clupeine from herring sperm, iridine from trout sperm, sturine from sturgeon sperm and scombrine from mackerel sperm. Protamine, protamine sulphate, protamine phosphate and sperm nucleii are readily available from Sigma-Aldrich.

Other suitable polyamines such as putrescine, spermidine and spermine, together with physiologically acceptable salts thereof are also readily available, for example, from Sigma-Aldrich.

The condensing agent may also comprise multivalent cations or their physiologically acceptable salts. Such multivalent cations include, for example, hexamine cobalt (III) chloride (Cohex), tris(ethylenediamine) cobalt (III) chloride (Coen) and cobalt (III) sepulchrate chloride (Cosep). Suitable physiological salts include those listed above in relation to cationic polymers.

A number of tests are known in the art which can be used to identify nucleic acid condensing agents. These generally assay for a change in the properties of a test nucleic acid molecule where the alteration is associated with the condensation process e.g compaction of the nucleic acid to a solid, neutralisation of the charge of the nucleic acid molecule, or obscuring or blocking of previously accessible recognition sites for agents such as endonucleases and transcription factors.

Assays, indicative of condensation for liquid nucleic acid formulations include but are not limited to: electron and atomic force microscopy, particle size,

zeta potential, spectrofluorometric, ethidium bromide exclusion assay, gel shift, circular dichroism and nuclear magnetic resonance. Examples of useful literature citations are: J. Mol Biol (1978) 121, 311-326; Biophysical Journal (1996), 70, 2847-2856, Nucleic Acids Res (1999) 27(8), 1943-1949; J. Amer. Chem. Soc., (1998) 120 (35), 8903-8909; J. Pharm Sci (1998) 87(6), 678-683 and Int. J. Pharm (2000) 210 (1-2), 97-107.

The chelating agent for use in the present invention chelates metal ions from solution. Commonly occurring metal ions for chelation include Fe^{2+}, Fe^{3+}, Cr^{3+}, Ca^{2+} and Na^{+} ions. Preferably, the agent chelates Ca^{2+} or Na^{+} ions. Alternatively, it is preferred that the agent chelates Fe^{2+} or Fe^{3+} ions. The agent may be mono- or multi-dentate. For example, suitable agents include but are not limited to ethylenediamine tetraacetic acid (EDTA) diethylenetriamine penta-acetic acid (DTPA), nitrilotriacetic acid (NTA), inositol hexaphosphate, tripolyphosphate, polyphosphoric acid, sodium succinate, potassium succinate, lithium succinate, sodium malate, potassium malate, lithium malate, desferal and ethylenediamine-di (o-hydroxy-phenylacetic) acid (EDDHA). Typically, EDTA, DTPA or desferal is used, in particular EDTA or a salt thereof, for example edetate disodium.

It is also preferred, that the nucleic acid is deposited on the inert metal carrier particles in the presence of one or more disaccharide, and/or trisaccharide sugars. The one or more sugars help to increase the stability of the nucleic acid/metal particle.

Suitable sugars include but are not limited to sucrose, sucrose monolaurate, trehalose, lactose, raffinose and mannitol. Preferably, the sugar is selected from trehalose, sucrose, lactose and raffinose.

In one embodiment, a blend of one or more disaccharide or trisaccharide sugars is used. For example, a blend of one or more of the sugars listed above may be used. A blend of sucrose/raffinose is particularly preferred. Typically, the sucrose/raffinose is blended at a ratio 3:1 wt:wt

Nucleic acid may be deposited on the inert metal carrier particle also in the presence of one or more salts. The one or more salts provide still further stability.

The salt is in addition to any salt which may be used according to the invention as a chelating agent. Therefore, the salt referred to is in general a non-chelating salt. For example the salt is typically not a malate or a succinate. The salt is also in addition to a physiologically acceptable salt which may be used according to the invention as a nucleic acid condensing agent.

In a preferred embodiment, the one or more salts is selected from chlorides, acetates, citrates, nitrates, phosphates and sulphates. Suitable salts include, but are not limited to potassium acetate, calcium chloride, lithium chloride, sodium acetate, magnesium nitrate, sodium citrate, sodium phosphate, sodium chloride, sodium sulphate and potassium sulphate. Preferably the salt is potassium acetate.

In a preferred embodiment, the nucleic acid bearing particles are contacted with an antioxidant such as ethanol or vitamin A, vitamin C or vitamin E. Typically, treating the particles with the antioxidant increases stability. A typical treatment may involve washing the particles with the antioxidant.

In one embodiment, one or more of the components used in particle preparation becomes incorporated in or associated with the resultant particle. Accordingly, there are provided particles, suitable for delivery from a particle-mediated delivery device, comprising or sometimes consisting essentially of inert metal carrier particles having on their surface a nucleic acid, a metal ion chelating agent and one or more of:

(i) a nucleic acid condensing agent;

(ii) one or more disaccharide and/or trisaccharide sugars; and

(iii) one or more salts.

Each of the particle components is as described above. Preferably the particles comprise at least (i) and /or (ii).

The invention also provides a process for the preparation of the present particles. The process comprises or in some embodiments consists essentially of

(i) precipitating a nucleic acid on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent; and

(ii) collecting the resultant particles;

Typically, step (i) comprises contacting the nucleic acid with the inert metal carrier particles in the presence of the nucleic acid condensing agent and the metal ion chelating agent by admixing. It is preferable to vortex the mix continually during the contacting procedure to ensure uniformity of the reaction mixture. Preferably the nucleic acid and nucleic acid condensing agent are separated from each other (eg in separate solutions) until the time when condensation is to occur for example until the carrier particles and chelating agent are also present. It is particularly preferred to add the condensing agent to a mix already comprising the carrier particles and the nucleic acid, to avoid premature precipitation of the nucleic acid. The metal ion chelating agent may then be present in either or both of the condensing agent preparation and the mix of carrier particles and nucleic acid. The condensing agent may be added stepwise, for example dropwise.

Alternatively, a mix of condensing agent and carrier particles may be admixed with a nucleic acid preparation.

The metal particles, nucleic acid, nucleic acid condensing agent and the chelating agent have been described above.

The mix in the precipitation step may additionally include one or more disaccharide and/or trisaccharide sugars, for example, sucrose, sucrose monolaurate, trehalose, lactose, raffinose or mannitol, or a combination thereof. Preferably the sugar is selected from trehalose, sucrose, lactose and raffinose or a combination thereof. A blend of one or more disaccharide and /or trisaccharide sugars may be used. For example a blend of one or more of the above sugars may be used. A blend of sucrose: raffinose is particularly useful, especially at a ratio of 3:1 wt:wt

The precipitation mix may also include one or more salts. The salt is in addition to any salt which may be used according to the invention as a chelating agent. Therefore the salt referred to is in general a non-chelating salt. For example the salt is typically not a malate or succinate. The salt is also in addition to any physiologically acceptable salt which may be used according to the invention as a nucleic acid condensing agent.

In a preferred embodiment, the one or more salts are selected from chlorides,

acetates, citrates, nitrates, phosphates and sulphates. Suitable salts include, but are not limited to potassium acetate, calcium chloride, lithium chloride, sodium acetate, magnesium nitrate, sodium citrate, sodium phosphate, sodium chloride, sodium sulphate and potassium sulphate. Typically the salt is not aluminum phosphate. Preferably the salt is potassium acetate.

The sugar/and or salt may be present in either or both of the initial preparations to be admixed, for the example a condensing agent preparation or a carrier particle/nucleic acid preparation.

In one embodiment, a solution comprising predetermined amounts of nucleic acid condensing agent, sugar and metal ion chelating agent is added dropwise to a solution comprising predetermined amounts of metal carrier particles, nucleic acid, sugar and metal ion chelating agent. Salt may be present in either or both solutions.

The concentrations of the components in step (i) may be varied without substantially affecting the stability of the nucleic acid in the resultant particles. The metal carrier particles may present in any suitable amount, for example in a suspension of from 0.1 to 100 mg/ml such as from 0.1 to 10 mg/ml or 1 to 10 mg/ml. Preferably, the nucleic acid concentration is from 0.01 to 10 mg/ml, such as from 0.1 to 1 mg/ml. Typically the condensing agent is at a concentration of from 0.1 to 10 mg/ml, for example, from 0.1 to 1 mg/ml. Preferably the metal ion chelating agent concentration is from 0.1 mM to 1 M, such as from 1 mM to 0.1 M for example 10 to 50 mM. Sugar, if present may be for example, at a concentration of from 0.1 mg/ml to 1 g/ml, preferably from 1 mg/ml to 0.1 g/ml, for example 10 to 50 mg/ml. Salt, if present, is typically at a concentration of from 0.1 mM to 1M for instance from 1 mM to 0.1 M, such as 10 to 50 mM.

The process of the invention may additionally comprise contacting the nucleic acid particles with an antioxidant. Generally this treatment involves washing the particles with a solution of the antioxidant. For example, ethanol may be used. Preferably the ethanol is sucrose saturated. Other suitable antioxidants include vitamin A, vitamin C and vitamin E. Additionally, or alternatively, the nucleic acid particle may be washed one or more times with an aqueous or alcoholic solution such

as water or isopropanol.

The coated, optionally washed particles can be transferred to suitable membranes and allowed to dry before use, coated onto surfaces of a sample module or cassette, or loaded into a delivery cassette for use in particular particle-mediated delivery instruments. Any suitable drying method may be used. Preferably drying is carried out under a nitrogen stream.

The particles of the invention may be packaged in single unit dosages or multidose containers. Such containers may comprise an hermetically sealed container enclosing a suitable amount of the particles. The particles can be packaged as a sterile formulation, and the hermetically sealed container can thus be designed to preserve the sterility of the formulation until use in delivery to a subject. The containers are preferably adapted for direct use in a particle mediated delivery device. Typically such containers take the form of capsules, foil pouches, sachets, cassettes and the like. The particle delivery devices can also be provided in a preloaded condition containing a suitable dosage of the coated particles. The preloaded device may then also be prepackaged in a hermetically sealed container.

The container in which the particles are packaged can further be labelled to identify the composition and provide relevant dosage information. In addition, the container can be labelled with a notice in the form prescribed by a governmental agency, for example, the Food and Drug Administration, wherein the notice indicates approval by the agency under Federal Law of the manufacture, use or sale of the nucleic acid preparation contained therein for human administration.

Particle acceleration devices, suitable for particle-mediated delivery are known in the art. Current gene gun devices employ an explosive, electric or gaseous discharge to propel coated carrier particles towards target cells. The coated carrier particles can be releasably attached to a movable carrier sheet, or removably attached to a surface along which a gas stream passes, lifting the particles from the surface and accelerating them toward the target. An example of a gaseous discharge device is described in U.S.Patent No. 5,204,253. An explosive-type device is described in U.S.Patent No. 4,945,050. One example of an electric discharge apparatus suitable

for use herein is described in U.S.Patent No. 5,120,657. Another electric discharge apparatus is described in US Patent No 5,149,655. The disclosure of all of these patents is incorporated herein by reference in their entireties.

The present coated particles may also be administered using a needleless syringe device, such as those described in U.S.Patent No. 5,630,796 to Bellhouse et al ("the PowderJect® needleless syringe device") and in International Publication Nos. WO 94/24263, WO 96/04947, WO 96/12513 and WO 96/20022, all of which are incorporated herein by reference.

Devices such as the one described in US Patent No.5,630,796 may be provided as a pen-shaped instrument containing, in linear order moving from top to bottom, a gas cylinder, a particle cassette or package, and a supersonic nozzle with an associated silencer medium. The particles are provided within a suitable container, e.g. a cassette formed by two rupturable polymer membranes that are heat-sealed to a washer-shaped spacer to form a self-contained sealed unit. Membrane materials can be selected to achieve a specific mode of opening and burst pressure that dictate the conditions at which the supersonic flow is initiated.

In operation, the device is actuated to release the compressed gas from the cylinder into an expansion chamber within the device. The released gas contacts the particle cassette and, when sufficient pressure is built up, suddenly breaches the cassette membranes sweeping the particles into the supersonic nozzle for subsequent delivery. The nozzle is designed to achieve a specific gas velocity and flow pattern to deliver a quantity of particles to a target surface of predefined area. The silencer is used to attenuate the noise produced by the supersonic gas flow.

The delivery system described in International Publication No. WO 96/20022 also uses the energy of a compressed gas source to accelerate and deliver powdered compositions. However, it is distinguished from the system of US Patent No. 5,630,796 in its use of a shock wave instead of gas flow to accelerate the particles. More particularly, an instantaneous pressure rise provided by a shock wave generated behind a flexible dome strikes the back of the dome, causing a sudden eversion of the flexible dome in the direction of a target surface. This sudden eversion catapults a

powdered composition (which is located on the outside of the dome) at a sufficient velocity, thus momentum, to penetrate target tissue, e.g., oral mucosal tissue. The powdered composition is released at the point of full dome eversion. The dome also serves to completely contain the high-pressure gas flow which therefore does not come into contact with the tissue. Because the gas is not released during this delivery operation, the system is inherently quiet. This design can be used in other enclosed or other wise sensitive applications for example, to deliver particles to minimally invasive surgical sites.

The present coated particles may be delivered *in vivo* directly to a subject, or *ex vivo* to cells taken from a subject, the transformed cells then being reimplanted in the subject. For *in vivo* delivery, particle injection is typically subcutaneously, epidermally, intradermally, intramucosally (e.g. nasally, rectally and/or vaginally), intraperitoneally, intravenously, orally or intramuscularly. Preferably, delivery is to terminally differentiated cells; however, the particles can also be delivered to non-differentiated, or partially differentiated cells such as stem cells of blood and skin fibroblasts. Most preferably, delivery is to skin epidermal cells.

The coated particles are administered to a subject in a manner compatible with the dosage formulation and in an amount that will be prophylactically and/or therapeutically effective. A "therapeutically effective amount" of the present particulate compositions will be sufficient to bring about treatment or prevention of disease or condition symptoms, and will fall in a relatively broad range that can be determined by routine trials. Generally the particles are delivered in an amount of from 0.001 to 1000μg, more preferably 0.01 to 10.0μg of nucleic acid per dose. However, the exact amount necessary will vary depending on the age and general condition of the individual being treated and the particular nucleotide sequence selected, as well as other factors. An appropriate effective amount can be readily determined through clinical testing. The "Physicians Desk Reference" and "Goodman and Gilman's The Pharmacological Basis of Therapeutics" are useful for the purpose of determining the amount needed.

C. Experimental

Below are examples of specific embodiments for carrying out the methods of the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.* amounts, temperatures, etc.) but some experimental error and deviation should, of course, be allowed for.

Example 1

A series of experiments were carried out to assess the effects of various sugars, chelating agents and other excipients/additives in three DNA/gold particle formulations: "DSEP" (Experiment A) "poly Arg" (experiment B) and "modified spermidine" (experiment C)

The various particles were assessed according to one or more of the following criteria:

(i) DNA yield on particles was measured by elution and by spectrophotometry at A260, or fluorometry

(ii) physical stability of DNA on particles was assessed by gel analysis or by HPLC;

(iii) agglomeration of particles was assessed by light micoscopy or by actuation into a gel;

(iv) expression activity of DNA on particles was assessed by measuring expression of a luciferase reporter gene in CHO cells following introduction of particles bearing this gene into the cells;

(v) immunopotency of particles was assessed by measuring antibody titres in sera of mice, the mice having been vaccinated with particles bearing DNA encoding an antigen to which the antibody specifically binds.

For each formulation, particles were rank ordered for the above criteria.

Experiment A: The DSEP Formulation

Particles were prepared according to the following formula:

Gold particle -DNA-Sugar-Salt- Other

The sugars tested were selected from sucrose monolaurate, mannitol trehalose, lactose, raffinose and sucrose monocaprate. The salts tested were selected from potassium acetate, calcium chloride, lithium chloride, sodium acetate, magnesium nitrate, sodium citrate, sodium phosphate, aluminum phosphate, sodium chloride, sodium sulphate and magnesium chloride.

Various combinations of the sugars and salts were used as set out below. The effect of a wash with sucrose saturated ethanol was also assessed.

Results are shown below, with the various sugars or salts rank ordered for each criterion from the most effective or desirable to the least effective. For example, the top ranking sugars for DNA yield result in relatively high DNA yields; the top ranking sugars for agglomeration result in relatively low levels of agglomeration.

(i) DNA yield on particles

Sugar: Sucrose-Sucrose monolaurate~Trehalose~Lactose~Raffinose>>> Sucrose monocaprate (no yield)

Salt: Potassium acetate~Calcium chloride~Lithium chloride~Sodium acetate~Magnesium nitrate>Sodium citrate~Sodium phosphate>>> Aluminium phosphate

Other: A wash with sucrose saturated ethanol does not remove DNA from the gold powder

The absence of any salt greatly lowers yield

(ii) Physical stability of DNA on particles

Sugar: Sucrose>Trehalose>Mannitol~Lactose~Raffinose>sucrose monolaurate>sucrose monocaprate

Salt: Potassium acetate~sodium acetate~sodium citrate~sodium phosphate>sodium chloride>sodium sulfate>lithium chloride
Other: A wash with sucrose saturated ethanol contributes to stability
Aluminium phosphate prevents precipitation

(iii) Agglomeration

Sugar: Sucrose monocaprate<Raffinose~Lactose~Sucrose monolaurate<Sucrose~Trehalose
Salt: Potassium acetate~Lithium chloride~sodium phosphate~sodium citrate~sodium chloride~sodium sulfate<calcium chloride~magnesium chloride<magnesium nitrate

(iv) Expression activity of DNA on particles

Sucrose/Sodium acetate~Potassium acetate/Raffinose~Sucrose/Magnesium nitrate~Potassium acetate/Sucrose monolaurate

(v) Mouse immunopotency

For real-time aged formulas stored at room temperature:
Sucrose/Sodium acetate~Spermidine/$CaCl_2$ nucleic acid particles at 3 months
Raffinose/potassium acetate~Sucrose monolaurate/potassium acetate~Spermidine/$CaCl_2$ nucleic acid particles at 1 month

Experiment B: The poly Arg formulation

Particles were prepared according to the following formula:

Gold particle - DNA -Sugar - Chelating agent- Polyarginine peptide.

The sugars tested were selected from trehalose, sucrose and raffinose. The chelating agents tested were selected from EDTA, DTPA and desferal (DFO). The

polyarginine peptides tested were selected from 13000 Mw polyarginine, $(Arg)_6$ and $(Arg)_4$. Various combinations of sugar, chelating agent and polyarginine were tested.

Results are shown below, again in rank order from the most desirable component to the least desirable or effective.

(i) DNA yield on particles

Sugars tested: Trehalose, Sucrose, Raffinose

Chelators: EDTA, DTPA, desferal (DFO)

Other: poly-Arg peptides, either 13,000 Mw, $(Arg)_6$, $(Arg)_4$

Yields for all combinations of the above exceed 50% of the theoretical yield.

(ii) Physical stability of DNA on particles

Sugar: Sucrose>Trehalose>Raffinose

Chelators: EDTA>DTPA>desferal (DFO)

Other: All poly-Arg peptides gave similar stabilities

(iii) Agglomeration

Not a problem for this formulation

(iv) Expression activity of DNA on particles

Trehalose/EDTA/$(Arg)_4$ ~ Trehalose/DTPA/$(Arg)_4$.

Particles prepared using DNA, gold particles and spermidine, showed a similar level of expression activity to these formulations.

(v) Mouse immunopotency

Sucrose/EDTA/$(Arg)_4$ > Trehalose/EDTA/$(Arg)_4$ ~ Trehalose/DTPA/$(Arg)_4$ ~ Sucrose/DTPA/$(Arg)_4$

Particles prepared with DNA, $CaCL_2$ gold particles and spermidine resulted in a similar immunopotency to the Trehalose/EDTA/$(Arg)_4$

particles.

Experiment C: The modified spermidine formulation

Particles were prepared according to the following formula:

Gold particle- DNA- Spermidine -Sugar- Salt - Other.

The sugars tested were selected from sucrose, trehalose and raffinose. Salts tested were selected from magnesium chloride, magnesium nitrate, calcium chloride, sodium sulphate, potassium sulphate and sodium bromide.

The effect of spermidine concentration, and of treating particles with particular water/alcohol solutions was also tested.

Results are shown below.

(i) DNA yield on particles

Sugars: Raffinose>Trehalose>Sucrose

Salts: $MgCl_2$~MgNO3~$CaCl_2$>Sodium sulfate~potassium sulfate~sodium bromide

Other: Spermidine concentration and alcohol/water % also affect yield.

(ii) Physical stability of DNA on particles

Sugar: Raffinose>Trehalose>Sucrose

Salts: $MgCl_2$>$MgNO_3$>$CaCl_2$> Sodium sulfate~potassium sulfate~sodium bromide

(iii) Agglomeration

Not a problem for this formulation

Example 2: Stability study of various DNA-coated particles

Coated particles were prepared using various ratios of protamine sulphate, EDTA, water and one of trehalose, sucrose or lactose. Particles were prepared

according to ten different ratios, as set out in the ten formulae in Table 1. The stability of particles prepared according to each of the ratios was tested at 4°C and 60°C and at 0, 7 and 14 day time points.

Method

For each ratio, Tubes A and B were prepared according to Table 1. The contents of Tubes A and B were vortexed at high speed for 10s, or until each solution was well mixed. While vortexing Tube A at medium speed, the contents of Tube B were added dropwise using a 5ml pipetman. Tube A was vortexed for a further 15s, and the contents then allowed to rest for 5 minutes. The supernatant in Tube A was removed and the remaining pellet washed once with 1ml 100% isopropanol. The contents of the tube were vortexed and pelleted. The isopropanol was removed and the pellet dried to a powder with nitrogen stream.

3-5mg of each of the particle preparations was weighed into 1.5ml microcentrifuge tubes for incubation at 4°C or 60°C. The stability of the DNA in each of the particle preparations was tested at 0, 7, and 14 day time points, by agarose gel electrophoresis and HPLC.

Results

The particle preparations showed similar stability throughout testing. Particles prepared according to "ratio 4" (Formula 4, Table 1) were chosen for further work.

Table 1. Experimental DOE used to optimize the sugar and EDTA concentations for preparation of particles. The numbers across the top of the table are the final concentration of the sugar and EDTA in each tube. The numbers inside the table are in units of µl added to the tubes of each component.

Final sugar (mg/ml)	50	50	50	50	150	150	150	150	100	100	100	100	25	25	175	175	100	100	100	100
Final EDTA (mg/ml)	1	1	5	5	1	1	5	5	.3	3	3	3	3	3	3	3	0	0	6	6

Formula number	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		**10**	
	tube a	**tube b**	**tube a**	**tube b**	**tube a**	**tube b**	**tube a**	**tube b**	**tube a**	**tube b**	**tube a**	**tube b**	**tube a**	**tube b**	**tube a**	**tube b**	**tube a**	**tube b**	**tube a**	**tube b**
gold (24R) (mg)	35		35		35		35		35		35		35		35		35		35	
DNA (mg/ml) 1	70		70		70		70		70		70		70		70		70		70	
protaminesulphate (mg/ml) 1		210		210		210		210		210		210		210		210		210		210
sugar (mg/ml) 500	35	35	35	35	105	105	105	105	70	70	70	70	17.5	17.5	122.5	122.5	70	70	70	70
edta (mM) 50	7	7	35	35	7	7	35	35	21	21	21	21	21	21	21	21	0	0	42	42
water	238	238	210	70	168	28	140	0	189	49	189	49	241.5	101.5	136.5	-3.5	210	70	168	28
Total volume	350	350	350	350	350	350	350	350	350	350	350	350	350	350	350	350	350	350	350	350

Example 3: Further stability studies based on particles prepared according to "ratio 4" (Formula 4)

Particles were prepared as in Example 2 and according to ratio 4 in Table 1, but with on a larger scale (350mg gold particles), and with either

(a) no disaccharide (control);or

(b) trehalose; or

(c) sucrose;or

(d) lactose.

The particles were incubated at 4°C and 60°C, and the stability of the DNA on the particles assessed after 8 weeks by agarose gel electrophoresis.

Example 4: Development of tetraarginine formulations

After trying a few formulae to verify that polyarginines of various lengths have the ability to precipitate DNA onto gold microparticles, an experiment was set up to investigate what polyarginine length to use by studying yields and stabilities. Also investigated were the choice of sugar and chelator, which have been shown to enhance stability in previous formulas.

Results showed that polyarginines of 4 and 6 monomers were better for stability than 13000 molecular weight polymer (about 80 monomers). The sugars and chelators studied performed comparatively. Trehalose and sucrose were both good sugars. EDTA and DTPA were both good chelators. The arginine tetramer was chosen due to its likely quicker dissociation with DNA as well its likelihood to form less degradation products than the 6-mer. The resultant formulae for further study were named TA101.1 (DNA, sucrose, EDTA), TA101.2 (DNA, sucrose, DTPA), TA101.3 (DNA, trehalose, EDTA), TA101.4 (DNA, trehalose, DTPA).

These four formulae were placed on a long-term stability and studied against each other and a Spermidine/$CaCl_2$ control (in which spermidine and calcium chloride were used to precipitate DNA onto gold microparticles) at 4, 25 and 40 degrees Celsius. Their stabilities are shown in Table 2. These formulae were also tested for biological activity in a mouse study and in a luciferase expression

experiments (the DNA encoded luciferase).

Table 2: 101 Series Rates of Decay (of SC band)

Formula	Lot #	Temp (°C)	k ($days^{-1}$)	half life (d)
spm/$CaCl_2$	2251-45	25	0.0121	57
TA101.1	2251-28-I	25	0.0043	161
TA101.2	2251-28-II	25	0.0092	75
TA101.3	2251-28-III	25	0.0045	154
TA101.4	2251-28-IV	25	0.0113	61
spm/$CaCl_2$	2251-45	40	0.0373	19
TA101.1	2251-28-I	40	0.0107	65
TA101.2	2251-28-II	40	0.0261	27
TA101.3	2251-28-III	40	0.0117	59
TA101.4	2251-28-IV	40	0.0421	16

These activity experiments showed that the 101 series were all active, expressive and about equal to Spermidine/$CaCl_2$. The stabilities of the formulas TA101.1 and TA101.3 were better than TA101.2 and TA101.4. This indicates that the chelator EDTA was more stabilizing than DTPA, but there was little difference between trehalose (3 and 4) and sucrose (1 and 2) as formulated into these powders. The formula compositions of the 101 series of tetraarginine formulas are shown in Table 3.

Table 3: 101 Series Compositions

Formula	Sat'd sugar (30% v/v)	Chelator, 5 mM	Ethanol %	$(Arg)_4$ mg/ml
TA101.1	Sucrose	EDTA	0	0.3
TA101.2	Sucrose	DTPA	0	0.3
TA101.3	Trehalose	EDTA	0	0.3
TA101.4	Trehalose	DPTA	0	0.3

Next, two experiments were conducted in which saturation levels of trehalose and EDTA were used as the formulation environment. Along with these screens were multiple ethanol levels. These formulations utilized the idea that nearly saturated solutions could precipitate more stabilizers onto the gold. The formulae could be made at different water levels and densities by varying the percent ethanol. The formulae made in these environments seemed more stable than the 101 series of formulae.

An experiment designed to address optimization of ethanol, EDTA, trehalose and tetraarginine levels was then initiated. This experiment was performed under a design that varied four components (tetraarginine, EtOH, EDTA, trehalose) across five levels in specified ranges. A DNA mix was utilized that included 10% luciferase-encoding DNA. This allowed for the study of both stability and activity in one experiment. The optimal formulae were chosen by considering the outcomes, as well as modelling the data to empirical quadratic equations that predict stability and expression outcomes across the whole range of the experiment. The resultant formulae for further study were named TA201.2, TA201.5, TA 201.11 and TA201.15.

The compositions of these four formulae are shown in Table 4. These compositions are the compositions of the formulation environments, i.e. not of final powders. Table 3 shows that these formulae utilize ethanol as a high percent of the solvent. The major difference between 201.5 and 201.11 is trehalose level, but tetraarginine level is also three times higher in 201.5. The only difference between 201.2 and 201.15 is the presence of EDTA in 201.15.

The formula TA201.5 is the optimal formula according to optimization software when expression and stability criteria were optimized simultaneously. TA201.11 is the optimal formula when just stability was optimized. The others are there for comparison. TA201.2 was the most expressive formula and is included in further studies to set a high bar in activity experiments. TA201.15 was the center point of the experiment and was formulated multiple times. This formula therefore had the most repeated stability and activity data, and served as a good anchor to see if

the trends repeated themselves when the formulae were studied further.

Table 4: 201 Series Compositions

Formula	Trehalose mg/ml	EDTA mM	Ethanol %	$(Arg)_4$ mg/ml
TA201.5	40.05	37.5	52.5	1.13
TA201.15	80.1	25	35	0.75
TA201.11	120.15	37.5	52.5	0.38
TA201.2	80.1	0	35	0.75

Formulae TA201.5, TA201.15 and TA201.11 were very stable. In fact, a 25°C rate of decay measurement could not be obtained after 6 months of ageing. This temperature is closest to the real condition that the formulae would experience during storage. The stability of TA201.5 was studied at higher temperatures. Comparing relative decay rates at higher temperatures indicates relative stability at lower temperatures. Table 5 shows stabilities of formulae at various temperatures.

Table 5: Stabilities of TA's at Various Temperatures

Lot #	Formula	Temp.	k (days^{-1})	half life (d)
2251-136-sp	spm/CaCl2	4	0.0052	133.3
2251-136-sp	spm/CaCl2	25	0.0366	18.9
2251-136-sp	spm/CaCl2	40	0.2264	3.1
2251-136-2	TA201.2	4	0.0071	97.6
2251-136-2	TA201.2	25	0.0229	30.3
2251-136-2	TA201.2	40	0.2247	3.1
2251-110-15	TA201.15	60	0.0437	15.9
2251-110-11	TA201.11	60	0.0161	43.1
2334-18-11	TA201.11	60	0.0235	29.5
2251-110-5	TA201.5	60	0.0362	19.1
2334-80-n	TA201.5	60	0.0275	25.3
2334-80-m	TA201.5	60	0.0252	27.6
2251-156-5.1	TA201.5	60	0.0528	16.4
2251-156-5.2	TA201.5	60	0.0625	14.8
2251-156-5.3	TA201.5	60	0.0504	13.9
2334-101-1	TA201.5	60	0.0744	9.3
2334-101-2	TA201.5	60	0.726	9.5
2334-101-3	TA201.5	60	0.0781	8.9
2334-101-4	TA201.5	60	0.0645	10.8
2334-101-5	TA201.5	60	0.0530	13.1
2334-101-6	TA201.5	60	0.0735	9.4
2334-101-7	TA201.5	60	0.0514	13.5
2334-101-8	TA201.5	60	0.0719	9.6
2334-101-9	TA201.5	60	0.0241	28.7
2334-101-10	TA201.5	60	0.0163	42.6
2251-136-5	TA201.5	40	0.0027	253.9
2251-183-5	TA201.5	40	0.0029	236.6

The physical skin toxicity of formulae TA201.5 and TA201.11 was assessed. No adverse reactions were observed. The activities of the proteins encoded by the DNA in a formula were studied. Luciferase expression was observed when a luciferase-encoding DNA was used. Table 6 shows the results of animal studies in which DNA encoding hepatitis B core antigen (Cag) and hepatitis B surface antigen (Sag) was employed.

Table 6: Animal Studies Summary

Animal study (mouse and time and temperature for which powders aged if appropriate)	SAg ELISA	Cag ELISA	Sag ELISPOT	Cag ELISPOT
M103	201.5 > Spm	201.5 ≥ Spm	201.5 ≤ Spm	n/a
M108	201.5 ≤ Spm	201.5 ≥ Spm	201.5 ≥ Spm	201.5 ≥ Spm
M110, 0 time	201.5 ≤ Spm*	201.5 ≥ Spm*	201.5 ≥ Spm*	201.5 ≥ Spm*
M110, 1 month @ 25°C	201.5 ≥ Spm*	201.5 = Spm*	201.5 = Spm*	201.5 > Spm*
M110, 3 months @ 25°C	201.5 ≥ Spm*	201.5 ≥ Spm*	201.5 ≥ Spm*	201.5 > Spm*
G014	201.5 ≥ Spm	201.5 = Spm	n/a	n/a
M114, 3 months @ 25°C	201.5 ≤ Spm*	201.5 ≤ Spm*	201.5 ≥ Spm*	201.5 ≥ Spm*
M114, 4 months @ 40°C	201.5 ≤ Spm*	201.5 ≥ Spm*	201.5 ≥ Spm*	201.5 ≥ Spm*
M114, 6 months @ 40°C	201.5 ≤ Spm*	201.5 ≥ Spm*	201.5 ≥ Spm*	201.5 ≥ Spm*
M116, 2 wks @ 60°C	201.5 < Spm	201.5 ≤ Spm	201.5 = Spm	201.5 = Spm

* These were fresh Spermidine/$CaCl_2$. There were also aged Spermidine/$CaCl_2$ in M110 and M114.

These data clearly indicate that formula TA201.5 was competitive with Spermidine/$CaCl_2$ in terms of antibody ELISA and ELISPOT response assays on ND5.5 transfected animals at a 2 μg DNA dose with a 1 mg of carrier particles load. The formula showed consistent greater or equal to Spermidine/$CaCl_2$ performance with respect to the ELISPOT data (by Mann-Whitney criteria).

The final composition of formula TA201.5 was measured. Table 7 shows the total composition ranges that have been measured from TA201.5 powders.

Table 7: TA201.5 Final Total Compositions and Ranges

Component	µg/mg powder	Chemical Formula, M.W.
gold micro particles	~1 mg	Au_n diameter ~ 2 µm
pDNA	~2	about 5-10K base pairs
H-Arg-Arg-Arg-Arg-OH	0.2-0.8	$C_{24}H_{50}N_{16}O_5$, 642.8 g/mol
*EDTA (4-)	0.2-1.2	$C_{10}H_{12}N_2O_8$, 290 g/mol
D(+)Trehalose	1.0-4.0	$C_{12}H_{22}O_{11}{}^*2H_2O$, 378.3 g/mol

* EDTA is measured in its 4(-) state, and is reported as a mass of this species (with no sodium, water or hydrogen as seen in the F.W. on the bottle).

Table 8 shows the materials that were used for the manufacture of tetraarginine formulations.

Table 8: Materials

Material	Supplier	Cat. #
pDNA	GSK, Aldevron, PJV, etc.	N/A
Tetaarginine	BaChem	H-4464
Na_2EDTA	Sigma	E-7889
Trehalose	Sigma	T-9531
Ethanol	Spectrum	ET107
H_20 (for sol'ns)	R.O.D.I.	N/A
10k gold particles	Degussa	RDAU010KM

The following procedure was used to formulate TA201.5 at a scale of 35 mg gold powder.

Equipment

Scale

Vortexer

Sonicator

Centrifuge

2 ml eppendorf tubes

1 ml, 200 µl pipetmen

Air stream

Preparation of Reagents

11.3 mg/ml tetraarginine (lot 523352): Weigh 0.8 to 1.0 mg tetraarginine. Add 88.5 µl H_20 per mg weighed. This will change for lots of different content.

Trehalose Solution: Weigh at least 30 mg trehalose (supplier shown in materials list). Add 120 µl H_20 per 30 mg weighed, or four times the amount of water than trehalose (in mass or µl, since 1 mg/µl).

500 mM Na_2EDTA: This can be ordered from Sigma in solution (see materials list for catalog number).

Procedure

Tube B: Have tube B ready before ethanol and DNA are added to tube A. Add the chelator solution, sugar solution, EtOH and tetraarginine solution. Vortex 10 seconds on high.

Tube A: Weigh the gold into the tube. Add the chelator solution and sugar solution. Sonicate 30 seconds, vortex 1 minute on high. Add the EtOH dropwise while vortexing. Add the DNA solution. After DNA is added, add tube B to tube A as described below.

Formulation Steps: While vortexing tube A on medium speed (making sure gold is being mixed throughout the solution) add the contents of tube B dropwise. After all of tube B has been added to tube A, vortex tube A, containing the final tetraarginine formulation, on high for 1 minute. Allow the formulation to settle for 5 minutes. Vortex, then centrifuge for 10 seconds. Pipette out the supernatant, and save it for analysis. Wash the pellet with 250 µl ethyl alcohol. Vortex for 30 seconds, sonicate for 3 seconds. Centrifuge the pellent down for 10 seconds and pull off the ethyl alcohol. Wash the pellet again with 250 µl ethyl alcohol. Vortex for 30

seconds, sonicate for 3 seconds. Centrifuge the pellet down for 10 seconds and pull off the ethyl alcohol. Dry the powder, using an air stream at a flow rate of 0.5 L/min, for 2 hours.

Tube A

- Gold powder, **35 mg**
- EDTA, **26.25 µl**
- Trehalose, **70 µl**
- Sonicate 30 sec, vortex 1 min
- EtOH, **183.75 µl dropwise vortexing**
- DNA, **70 µl**

Tube B

- EDTA, **26.25 µl**
- Trehalose, **70 µl**
- tetraarginine, **70 µl**
- EtOH, **183.75 µl**
- Vortex 10 sec

Tube A & Tube B

☺ Add tube B to tube A dropwise while vortexing

☺ Vortex for 1 minute

☺ Allow to settle for 5 minutes

☺ Centrifuge 10 sec and remove supernatant

☺ Wash with Ethyl Alcohol, **250 µl**, vortex 30 sec, sonicate 3 sec, centrifuge 10 sec, remove

☺ Wash with Ethyl Alcohol, **250 µl**, vortex 30 sec, sonicate 3 sec, centrifuge 10 sec, remove

☺ Dry under air or nitrogen for 2 hours

Throughout the development of formula TA201.5, the formulation procedure had been to first make individual stock solutions of each component, then add them to the formulation tubes in a certain order. However, clean-room manufacture of the formula would require a sterile filtration step. A "master mixing" experiment was initiated to address this necessity (NB2334-80). This strategy utilized master mixes of all the aqueous components in tube A (master A) and in tube B (master B). The

mixes were then syringe-filtered to sterilize, and the filtered material went into the formulation tubes. The master mixing experiment compared powders made by the master mix procedure and the standard procedure to each other in terms of stability and total composition.

Table 9: Final Compositions in Master Mix Experiment

Powder	DNA yield (%)	Trehalose (µg/mg)	EDTA (µg/mg)	ARG_4 (µg/mg)
Normal-2334-N	96.2	3.2	1.05	0.48
Master-2334-M	96.3	3.2	0.97	0.45

Table 10: Stabilities of Master vs. Normal

5-21-02, t=18 days							
Formula	ng OC	ng L	Ng SC	Total	% SC	% SC 60/ % SC 4	$t_{1/2}$
Normal, 4	6.1	0.7	43.5	50.3	86.5		
Normal, 60	14.5	0.5	23.1	38.1	60.7	0.70	25.3
Master, 4	6.3	0.8	49.2	56.2	87.5		
Master, 60	24.6	1.0	31.5	57.1	55.2	0.63	27.6

- Half lives in Table 10 were calculated by averaging two half-life calculations. One was derived from the ng SC rate of decay (60 relative to 4), and the other was derived from the % SC rate of decay (60 relative to 4).

Since the two procedures produced the same final product, the master mixing strategy could be considered non-problematic, and could be used for powder formulation of subsequent TA201.5 powders. A further process experiment was performed in order to decide what process steps were critical:

Experimental: Formulae 1-9 were made at a 70 mg scale from the same stock solutions. A master mix for tube A was made by mixing EDTA, trehalose, and DNA solutions in formulation ratios and then syringe filtered. Master mix for tube B

was achieved by mixing EDTA, trehalose, and tetraarginine solutions in formulation ratios, then syringe filtered. Formula 1 was then made by the following process:

Preparation of Reagents (for 10, *70 mg* prep)

DNA: Stocks of plasmid and luciferase-encoding DNA were provided at 1 mg/ml. Mix the two DNA solutions at a 9SC18 : 1 luciferase volume ratio.

1500 µl of SC18 + 166.7 µl of luciferase DNA.

11.3 mg/ml tetraarginine: Weigh ~20 mg tetraarginine. Add 88.5 µl H_20 per mg weighed.

Trehalose Solution: Weigh at least 700 mg trehalose. Add 140 µl H_20 per 35 mg weighed, or four times the amount of water than trehalose (in mass or µl, since 1 mg/µl).

500 mM EDTA: This can be ordered from Sigma in solution (Cat. E-7889).

Master A Volume Ratios: 1500 µl trehalose, 562. 5 µl EDTA, 1500 µl DNA (1 mg/ml)

Syringe filter Master A

Master B Volume Ratios: 1500 µl trehalose, 562.5 µl EDTA, 1500 µl tetraarginine

Syringe filter Master B

Tube A	Tube B
70 mg gold	
332.5 µl of master mix A	332.5 µl of master mix B
367.5 µl of EtOH dropwise while vortexing	367.5 µl of EtOH
Sonicate 30 sec, vortex 10 sec	vortex 10 sec

Tube A & Tube B

Add tube B to tube A dropwise while vortexing

After all of B is added, vortex for 1 minute on high

Allow to settle for 5 minutes

Vortex for 5 sec, then centrifuge for 10 sec, *remove supernatant

Wash with EtOH, 500 µl, vortex 30 sec, sonicate 3 sec, centrifuge 10 sec, *remove

Repeat the last step 1 time

Dry the pellet under air for 1 hour at 0.5 L/min

This process is the control formulation. The following Table shows the process variations of powders 2-9.

Variant step	Formula	Variation	Control
Add'n of B to A	2	All of B to A in one squirt	B to A dropwise/ continuous
	3	B to A dropwise/pause 5 sec between drops	
Vortex after B to A	4	3 minute vortex on high	1 minute vortex on high
	5	No vortex, allow to settle after all of B added to A	
Settling time	6	10 minute settle	5 minute settle
	7	No settling, centrifuge after 1 minute vortex	
Number of Washes	8	4 washes	2 washes
	9	No washes, dry after supernatant removal	

The remaining formulation was a formula in which the order of addition was changed to assess the possibility of 1 master mix for this formulation. The process was similar to the control.

Master Mix Volume Ratio: 400 µl trehalose, 150 µl EDTA, and 200 µl tetraarginine

Syringe filter Master Mix

Formula 10

70 mg gold

525 µl of master mix

735 µl of EtOH dropwise while vortexing

Sonicate 30 sec, vortex 10 sec

140 µl of DNA dropwise/slowly (pause 5 sec between drops) while vortexing

After all of B is added, vortex for 1 minute on high

Allow to settle for 5 minutes

Vortex for 5 sec, then centrifuge for 10 sec, *remove supernatant

Wash with EtOH, 500µl, vortex 30 sec, sonicate 3 sec, centrifuge 10 sec, *remove

Repeat the last step 1 time

Dry the pellet under air for 1 hour at 0.5 L/min

* Removals of liquid from the pellet after centrifugation will be done with a 1000 µl pipette to remove most of the liquid, followed by a 200 µl pipette to remove the rest of the liquid. As little as possible will be left on the powder.

All of the powders were analysed in terms of total compositions, stability, luciferase expression in CHO cells, and gel shots.

Table 11: Compositions of Process Robustness Experiment

Powder	$(Arg)_4$ (µg/mg)	Trehalose (µg/mg)	EDTA (µg/mg)
2334-101-1	0.36	1.56	0.48
2334-101-2	0.32	1.58	0.47
2334-101-3	0.36	1.72	0.46
2334-101-4	0.29	1.64	0.39
2334-101-5	0.22	1.72	0.41
2334-101-6	0.26	1.54	0.41
2334-101-7	0.26	2.13	0.40
2334-101-8	0.26	1.15	0.19
2334-101-9	0.23	4.77	0.99
2334-101-10	0.21	2.25	0.40

Table 12: Stabilities at 60°C of Process Robustness Experiment

Formula	K	t1/2 (days)
1	0.0744	9.31
2	0.0726	9.55
3	0.0781	8.88
4	0.0645	10.75
5	0.0530	13.07
6	0.0735	9.43
7	0.0514	13.50
8	0.0719	9.64
9	0.0241	28.75
10	0.0163	42.61

- The above numbers were calculated by changes in nano-grams of super-coiled DNA from the date of formulation to 13 and 20 days.

The rates and half-lives were very close to each other. This indicates that the formulation process is robust in terms of stability. Formulae 5 and 7 had slightly

better stabilities than the others, but formulae 9 and 10 had a distinct stability advantage (3X). Formula 9 was not washed (and not feasible to process) and formula 10 was achieved by a change in order of additions.

The expression data indicated that the formulae were very similar (300-400K counts/sec). The fresh Spermidine/$CaCl_2$ formula were more expressive (or had more expressed material at the time the cells were analyzed) but also measured higher in release mass. Formulae 10 and 7 had some luciferase expression advantage (450-600K counts/sec) over the other tetraarginine formulae. Overall, the experiment proved the process to be very robust.

Another aspect of robustness is the effect of altering the orders of additions and processes used to make a formula. In one such experiment (NB2251-124) different ways of making a formula with the same exact materials (amounts and concentrations) were investigated. This experiment only looked at stabilities of formulas designed to precipitate components in different orders. This could presumably have large effects since DNA could be precipitated directly to gold before, simultaneously, or after stabilizers.

Formulation Processes:

1. Tubes A and B approach. Tubes A and B have the same composition, except A has DNA, and B has tetraarginine. Both are well mixed, and then B is added to A dropwise.

2. Another tubes A and B approach. This time, in tube A (with gold), the ethanol was added before the DNA sol'n in order to pre-coat the gold with an excess amount of sugar before DNA addition. Then DNA was added to tube A, and tube B was then added to tube A immediately.

3. This approach was designed to simultaneously precipitate DNA and stabilizers. All components were added to one tube except DNA and ethanol. Then, the DNA and ethanol were mixed together and added.

4. This process precipitated DNA prior to ethanol addition. All

components were added to one tube except DNA, tetraarginine and ethanol. The order of addition of these last components was DNA, tetraarginine and ethanol respectively.

5. This process was the same as 4 except tetraarginine was added before DNA. Ethanol was still last.

These formulations were placed in 4 and 60 degree incubators, and analyzed 2 weeks after formulation. There were no gross differences between these powders by agarose gel electrophoresis, although powders 4 and 5 (ethanol last, DNA:ARG4 complexation before ethanol) were slightly more stable.

Accordingly, novel nucleic acid coated particles suitable for delivery from a particle-mediated delivery device, a process for preparing the particles, and methods of gene therapy and nucleic acid immunisation using the particles have been described. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and scope of the invention as defined in the appended claims.

CLAIMS

1. Particles suitable for delivery from a particle-mediated delivery device, which particles are obtainable by precipitating a nucleic acid on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent.

2. Particles according to claim 1 wherein the inert metal carrier particles are selected from the group consisting of gold, tungsten, platinum and iridium particles.

3. Particles according to claim 2 wherein the inert metal carrier particles are gold particles having a diameter from about 1 to 3μm.

4. Particles according to any one of the preceding claims wherein the nucleic acid encodes an antigen.

5. Particles according to claim 4 wherein the antigen is selected from the group consisting of viral antigens, bacterial antigens and fungal antigens.

6. Particles according to any one of claims 1 to 3 wherein the nucleic acid encodes a therapeutic polypeptide.

7. Particles according to any one of the preceding claims wherein the nucleic acid is DNA.

8. Particles according to any one of the preceding claims wherein the nucleic acid condensing agent is a cationic polymer.

9. Particles according to claim 8 wherein the nucleic acid condensing agent is a polyamine.

10. Particles according to claim 9 wherein the polyamine is selected from the group consisting of protamines, spermidine, spermine, putrescine, and physiologically acceptable salts thereof.

11. Particles according to claim 9 wherein the polyamine is a polyarginine or a polylysine.

12. Particles according to claim 11 wherein the polyarginine is $(Arg)_4$ or $(Arg)_6$.

13. Particles according to any one of the preceding claims wherein the metal ion chelating agent is selected from the group consisting of ethylenediamine tetraacetic acid (EDTA) diethylenetriamine penta-acetic acid (DTPA), nitrilotriacetic acid (NTA), inositol hexaphosphate, tripolyphosphate, polyphosphoric acid, sodium succinate, potassium succinate, lithium succinate, sodium malate, potassium malate, lithium malate, desferal and ethylenediamine-di (o-hydroxy-phenylacetic) acid (EDDHA).

14. Particles according to any one of the preceding claims wherein precipitation is carried out in the presence of one or more disaccharide and/or trisaccacharide sugars.

15. Particles according to claim 14 wherein the one or more sugars is selected from the group consisting of trehalose, sucrose, lactose and raffinose.

16. Particles according to claim 15 wherein the one or more sugars is a blend of sucrose and raffinose.

17. Particles according to any one of the preceding claims wherein precipitation is carried out in the presence of one or more salts.

18. Particles according to claim 17 wherein the one or more salts is selected from the group consisting of potassium acetate, calcium chloride, lithium chloride, sodium acetate, magnesium nitrate, sodium citrate, sodium phosphate and magnesium chloride.

19. Particles according to any one of the preceding claims wherein the resultant particles are contacted with an antioxidant.

20. Particles according to claim 19 wherein the antioxidant is selected from the group consisting of ethanol, vitamin A, vitamin C and vitamin E.

21. Particles according to claim 1 which have been obtained by precipitating DNA on gold carrier particles in the presence of a polyarginine, EDTA and sucrose.

22. A dosage receptacle for a particle-mediated delivery device, the receptacle containing particles which are obtainable by precipitating a nucleic acid on

inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent.

23. A particle mediated delivery device loaded with particles which are obtainable by precipitating a nucleic acid on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent.

24. A particle mediated delivery device according to claim 23 which is a needleless syringe.

25. A process for the preparation of particles suitable for delivery from a particle-mediated delivery device, comprising the steps of:

(i) precipitating a nucleic acid on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent; and

(ii) collecting the resultant particles.

26. A process according to claim 25 wherein, in step (i) the nucleic acid condensing agent is added to a mixture comprising the inert metal carrier particles and the nucleic acid.

27. A process according to claim 25 or 26 wherein the inert metal carrier particles are selected from the group consisting of gold, tungsten, platinum and iridium particles.

28. A process according to claim 27 wherein the inert metal carrier particles are gold particles having a diameter from about 1 to 3μm.

29. A process according to any one of claims 25 to 28 wherein the nucleic acid encodes an antigen.

30. A process according to claim 29 wherein the antigen is selected from the group consisting of viral antigens, bacterial antigens and fungal antigens.

31. A process according to any one of claim 25 to 28 wherein the nucleic acid encodes a therapeutic polypeptide.

32. A process according to any one of claims 25 to 31 wherein the nucleic acid is DNA.

33. A process according to any one of claims 25 to 32 wherein the nucleic acid condensing agent is a cationic polymer.

34. A process according to claim 33 wherein the nucleic acid condensing agent is a polyamine.

35. A process according to claim 34 wherein the polyamine is selected from the group consisting of protamines, spermidine, spermine, putrescine and physiologically acceptable salts thereof.

36. A process according to claim 34 wherein the polyamine is a polyarginine or a polysine.

37. A process according to claim 36 wherein the polyarginine is $(Arg)_4$ or $(Arg)_6$.

38. A process according to any one of claims 25 to 37 wherein the metal ion chelating agent is selected from the group consisting of ethylenediamine tetraacetic acid (EDTA) diethylenetriamine penta-acetic acid (DTPA), nitrilotriacetic acid (NTA), inositol hexaphosphate, tripolyphosphate, polyphosphoric acid, sodium succinate, potassium succinate, lithium succinate, sodium malate, potassium malate, lithium malate, desferal and ethylenediamine-di (o-hydroxy-phenylacetic) acid (EDDHA).

39. A process according to any one of claims 25 to 38 wherein step (i) is further carried out in the presence of one or more disaccharide and/or trisaccharide sugars.

40. A process according to claim 39 wherein the one or more sugars is selected from the group consisting of trehalose, sucrose, lactose and raffinose.

41. A process according to claim 40 wherein the one or more sugars is a blend of sucrose and raffinose.

42. A process according to any one of claims 25 to 41 wherein step (i) is further carried out in the presence of one or more salts.

43. A process according to claim 42 wherein the one or more salts is selected from the group consisting of potassium acetate, calcium chloride, lithium chloride, sodium acetate, magnesium nitrate, sodium citrate, sodium phosphate and magnesium chloride.

44. A process according to any one of claims 25 to 43 wherein the resultant particles from step (i) are contacted with an antioxidant.

45. A process according to claim 44 wherein the antioxidant is selected from the group consisting of ethanol, vitamin A, vitamin C and vitamin E.

46. A process according to claim 25 comprising the steps of:

(i) precipitating DNA on inert gold particles in the presence of a polyarginine, EDTA and sucrose; and

(ii) collecting the resultant particles.

47. A method of nucleic acid immunisation comprising

(a) providing particles suitable for delivery from a particle-mediated delivery device, which particles are obtainable by precipitating a nucleic acid encoding an antigen on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent; and

(b) administering an effective amount of the particles to a subject.

48. A method of gene therapy comprising

(a) providing particles suitable for delivery from a particle-mediated delivery device which particles are obtainable by precipitating a nucleic acid encoding a therapeutic polypeptide on inert metal carrier particles in the presence of a nucleic acid condensing agent and a metal ion chelating agent; and

(b) administering an effective amount of the particles to a subject .

49. A method according to claim 47 or 48 wherein the inert metal carrier particles are selected from the group consisting of gold, tungsten, platinum and iridium particles.

50. A method according to claim 49 wherein the inert metal carrier particles are gold particles having a diameter from about 1 to 3μm .

51. A method according to any one of claims 47 to 50 wherein the nucleic acid is DNA.

52. A method according to any one of claims 47 to 51 wherein the nucleic acid condensing agent is a cationic polymer.

53. A method according to claim 52 wherein the nucleic acid condensing agent is a polyamine.

54. A method according to claim 53 wherein the polyamine is selected from the group consisting of protamines, spermidine, spermine, putrescine and physiologically acceptable salts thereof.

55. A method according to claim 53 wherein the polyamine is a polyarginine or a polylysine.

56. A method according to claim 55 wherein the polyarginine is $(Arg)_4$ or $(Arg)_6$.

57. A method according to any one of claims 47 to 56 wherein the metal ion chelating agent is selected from the group consisting of ethylenediamine tetraacetic acid (EDTA) diethylenetriamine penta-acetic acid (DTPA), nitrilotriacetic acid (NTA), inositol hexaphosphate, tripolyphosphate, polyphosphoric acid, sodium succinate, potassium succinate, lithium succinate, sodium malate, potassium malate, lithium malate, desferal and ethylenediamine-di (o-hydroxy-phenylacetic) acid (EDDHA).

58. A method according to any one of claims 47 to 57 wherein precipitation is carried out in the presence of one or more disaccharide and/or trisaccharide sugars.

59. A method according to claim 58 wherein the one or more sugars is selected from the group consisting of trehalose, sucrose, lactose and raffinose.

60. A method according to claim 59 wherein the one or more sugars is a blend of sucrose and raffinose.

61. A method according to any one of claims 47 to 60 wherein precipitation is carried out in the presence of one or more salts.

62. A method according to claim 61 wherein the one or more salts, is selected from the group consisting of potassium acetate, calcium chloride, lithium chloride, sodium acetate, magnesium nitrate, sodium citrate, sodium phosphate and magnesium chloride.

63. A method according to any one of claims 47 to 62 wherein the resultant particles are contacted with an antioxidant.

64. A method according to claim 63 wherein the antioxidant is selected from the group consisting of ethanol, vitamin A, vitamin C and vitamin E.

65. A method according to claim 47 comprising the steps of:

(a) providing particles suitable for delivery from a particle-mediated delivery device, which particles have been obtained by precipitating DNA encoding an antigen on gold particles in the presence of a polyarginine, EDTA and sucrose; and

(b) administering an effective amount of the particles to a subject.

66. A method according to claim 48 comprising the steps of:

(a) providing particles suitable for delivery from a particle-mediated delivery device, which particles have been obtained by precipitating DNA encoding a therapeutic polypeptide on gold particles in the presence of a polyarginine, EDTA and sucrose; and

(b) administering an effective amount of the particles to a subject.

67. Particles, suitable for delivery from a particle mediated delivery device, which comprise inert metal carrier particles having on their surface a nucleic acid, a metal ion chelating agent and one or more of :

(i) a nucleic acid condensing agent;

(ii) one or more disaccharide and/or two trisaccharide sugars; and

(iii) one or more salts.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 03/04202

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/00 A61K9/00 A61K38/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 204 253 A (BRUNER RONALD F ET AL) 20 April 1993 (1993-04-20) column 15; claims 1-35 column 23	1-10,13, 17, 19-23, 25-35, 38, 42-45, 47-54, 57,61-64
A	US 4 945 050 A (SANFORD JOHN C ET AL) 31 July 1990 (1990-07-31) The whole document	1-67
Y	US 6 288 312 B1 (CHRISTOU PAUL ET AL) 11 September 2001 (2001-09-11) column 6 -column 8; claim 1	1-67
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 2004	02/02/2004

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB 03/04202

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 630 796 A (BELLHOUSE BRIAN J ET AL) 20 May 1997 (1997-05-20) cited in the application claims 1-14; examples 1-3	1-67
Y	WO 00 26385 A (POWDERJECT VACCINES INC) 11 May 2000 (2000-05-11) page 22-25 page 31 page 32; examples 2-4	1-67
Y	WO 01 83528 A (POWDERJECT RES LTD ;POWDERJECT VACCINES INC (US)) 8 November 2001 (2001-11-08) page 24 -page 32; claims 1,15-18,59,67	1-67
A	FYNAN E F ET AL: "DNA VACCINES: PROTECTIVE IMMUNIZATIONS BY PARENTERAL, MUCOSAL AND GENE-GUN INOCULATIONS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 90, no. 24, 15 December 1993 (1993-12-15), pages 11478-11482, XP000572203 ISSN: 0027-8424 Abstract, page 2, Discussion	1-67

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 03/04202

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 47-65 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB 03/04202

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EXHIBIT B

case of the European cod. The diet of the adult hake is comprised of rattails, maasbanker, and squid, and cannibalism is quite common.

See also **Fishes.**

HALF-ADDER (Computer System). A circuit having two output points, *S* and *C*, representing sum without carry and carry, and two input points, *A* and *B*, representing addend and augend, such that the output is related to the input according to the following table:

Input		Output	
A	*B*	*S*	*C*
0	0	0	0
0	1	1	0
1	0	1	0
1	1	0	1

Two half-adders and an Inclusive-OR circuit, properly connected, can provide a Full-Adder having two inputs (augend and addend) and a carry input which produces a sum output (without carry) and a carry output. See **Full-Adder (Computer System).**

See also terms listed under **Data Processing.**

HALF-CELL. An electrochemical system consisting of a single electrode and an electrolytic solution, with usually a (reversible) ionization process in progress between electrode and electrolyte. See also **Galvanic Cell.**

HALF-LIFE (Biological). The time of survival of half the individual members of an unstable system. The half-life $t_{1/2}$ of the system is related to the decay constant λ and the mean life τ by the relation:

$$t_{1/2} = \frac{\ln 2}{\lambda} = \frac{0.693}{\lambda} = 0.693\tau$$

The term half-life is most commonly applied to systems of radionuclides but may also be applied to other systems that decay.

The biological half-life of a substance is the time in which a living tissue, organ or individual eliminates, through biological processes, one-half of a given amount of a substance which has been introduced into it. The effective half-life is a term usually applied to a radioactive substance in a biological organism. It is defined in terms of the half-life of the radioactive substance itself, and its biological half-life in the organism, by the following expression:

$$\text{effective half-life} = \frac{\text{radioactive half-life} \times \text{biological half-life}}{\text{radioactive half-life} + \text{biological half-life}}$$

HALF-SHADE PLATE. A semicircular, half-wave plate of quartz set between the polarizer and analyzer and close to the former. Useful in making precision settings with a polariscope.

HALF-LIFE (Elements). Chemical Elements.

HALF-SILVERED SURFACE. A surface coated with a metallic film of such thickness that it transmits approximately half of the light falling on it at normal incidence and reflects approximately half.

HALF-THICKNESS (Absorber). The thickness of a particular absorber that will reduce the intensity of a beam of radiation to one-half its initial value. If the absorption is exponential, the half-thickness is related to the linear or mass absorption coefficient and the mean free path as follows:

$$d_{1/2} = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} = 0.693l$$

where $d_{1/2}$ is the half-thickness, μ is the absorption coefficient and l is the mean free path.

of the contribution to the intensity betwe
width of the line is the halfwidth of the fi

HALIBUT. Flatfishes.

HALIDES. A compound made up of a h
chlorine, fluorine, or iodine) and another
termed a *halide*. Fundamentally, there are
(saline) halides, (2) the *covalent* (acid) hal
halides. The ionic halides are most sharply c
of the alkali and alkaline earth metals, plus t
and Actinide metals. They form ionic or
solid state, have high boiling points and melt
in polar solvents. Their bonding is electro
with the difference between the electronegat
the metal. Potassium iodide and silver fluo
iodide is essentially covalent. The fluorides
character for most of the metals, but the ot
ionic compounds. The degree of ionicity vari
the periodic table.

The covalent (acid) halides have low boi
are soluble in nonpolar solvents and insolut
though they often react with the latter. The d
ally is greatest for the nonmetals. For a give
point depends upon both the number of ato
which it is combined and the symmetry of the
the boiling points of bromine(I) fluoride, bron
bromine(V) pentafluoride, BrF, BrF_3 and B
40.5°C, respectively.

The complex halides are very numerous, b
with which halide ions form coordination co
In general, stability of these complexes depe
electronic structure of the metal ion—the sma
more stable compounds with the smaller hal
fluoride, while with larger cations the order
polarizability of the halide, i.e., decreasing fr
The more electronegative transition elements
complexes; e.g., those of palladium, platinum,
etc. The most common halo complexes have fo
coordinated with the cation, although such c
copper, gold and mercury, e.g., CuI_2^-, $AuCl_2^-$, H
exceptions.

See also **Bromine; Carbon; Chlorine; Chlorin
rine; and Iodine.**

HALITE (Rock Salt). The mineral halite (ro
occurring sodium chloride, NaCl, common salt.
cubic habit and cleavage. It is brittle; hardness,
2.168; luster, vitreous; colorless when pure, but u
red, or blue. It is soluble in water. Halite occu
sedimentary rocks in all parts of the world and
oldest rocks. It frequently occurs in association
gypsum. In the United States this type of "salt bed
in Michigan, New York, Ohio, and Pennsylvania.
salt from great subsurface dome-shaped masses
feet thick. The salt domes of the Gulf Coastal Pl
important as subsurface structures, on the flank
to occur large and important pools of petroleu
Austria, and France possess well-known deposits
the U.S.S.R., England, Algeria, India, and China.
in cooking and as a preservative; in the manufact
the glass industry; and as a source of many sodi
derives its name from the halogen group of element
belongs.

See also **Sodium Chloride.**

HALL EFFECT.

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EXHIBIT C

Stability of Peptide-Condensed Plasmid DNA Formulations

ROGER C. ADAMI,[†] WENDY T. COLLARD,[†] SHAMITA A. GUPTA,[†] KAI Y. KWOK,[†] JEFFREY BONADIO,[‡] AND KEVIN G. RICE*,[†]

Contribution from *Divisions of Pharmaceutics and Medicinal Chemistry, College of Pharmacy and Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109-1065.*

Received January 30, 1998. Accepted for publication March 27, 1998.

Abstract □ Low molecular weight homogeneous peptides were used to form peptide/DNA condensates. A peptide possessing 18 lysines was found to protect plasmid DNA from serum endonuclease and sonicative-induced degradation whereas a shorter peptide possessing 8 lysines dissociated in 0.1 M sodium chloride and failed to protect DNA from enzymatic degradation. Peptide-condensed DNA showed no change in the ratio of supercoiled to circular DNA following 100 W sonication for up to 60 s and was able to transfect HepG2 cells with equivalent efficiency as untreated condensed plasmid DNA. Alternatively, uncondensed plasmid DNA was rapidly fragmented by sonication and serum endonucleases and resulted in negligible gene expression following condensation with peptide. Cationic lipid/DNA complexes were only partially effective at stabilizing DNA in serum compared to the complete stabilization afforded by peptide/DNA condensation. These results indicate that the stabilization afforded by condensation with a peptide protects DNA during formulation and preserves its structure in serum. These functions are important to achieve optimal gene expression from a nonviral gene delivery system.

Introduction

The development of optimized plasmid DNA dosage forms for gene therapy requires an understanding of DNA stability during formulation and during transport to the target site.[1] To accomplish optimal and prolonged gene expression the delivery vehicle plays an important role in protecting DNA from physical, chemical, and enzymatic destruction. Delivery vehicles that bind to DNA and facilitate transport to cellular targets include peptides,[2] glycopeptides,[3] glycosylated polymers,[4,5] cationic lipids,[6-10] liposomes,[11,12] and PLGA microspheres.[13]

During fabrication of a delivery system the structure of DNA can be altered by shear stress to convert supercoiled plasmid to open circular, linear, or even fragmented DNA.[8] Shear stress generated by ultrasonication is a common approach used to minimize the particle size of a colloidal drug delivery system. Despite its ability to fragment DNA, several previous studies have used sonication as a means to increase the uptake of DNA by cells or to reduce particle size of colloidal DNA delivery systems.[14-16] The transfer of plasmid DNA into yeast cells in culture was reported to be improved 20-fold using mild sonication (2 W) for 30 s although further sonication led to significant DNA fragmentation and a decrease in the transfection efficiency.[14] Higher intensity sonication was reported to improve the encapsulation efficiency of DNA into liposomes, but the stability of the encapsulated plasmid DNA was only examined by low resolution gel permeation chromatography.[15] A cationic lipid/DNA formulation was also recently reported to be stable to 50 W sonication for 60 s to reduce particle size heterogeneity.[16]

Likewise, serum endonucleases can act in vivo during transport to fragment unprotected DNA and thereby abolish its ability to transform cells. Only a few studies have examined the role of the drug delivery vehicle in stabilizing DNA formulations in serum.[1,6,17] Uncomplexed plasmid DNA was found to be rapidly fragmented within 5 min in vivo and within 30 min during in vitro incubation in mouse serum due to the action of endogenous endonucleases.[1,17] Certain cationic lipid/DNA complexes were reported to partially protect DNA against purified DNAse I and against DNAase in human serum despite reports that cationic lipid gene transfer is greatly reduced in the presence of serum.[6,18]

Gel electrophoresis is still the most direct and sensitive approach to examine the stability of plasmid DNA in a gene delivery formulation or in serum. However, peptide and lipid delivery vehicles remain bound to DNA and interfere with its migration thereby necessitating phenol/chloroform extraction of DNA prior to electrophoresis. Other methods of analyzing DNA stability, such as gel permeation chromatography or the transformation of *Escherichia coli* or mammalian cells with extracted DNA, are less definitive compared to the direct analysis of DNA stability by gel electrophoresis since subtle alterations in DNA structure may escape detection.[14-16]

The stability profile of DNA formulations containing peptides and glycopeptides has not been reported previously. Partly, this is because of the difficulty of recovering DNA from tightly bound peptides which inhibit migration on gel electrophoresis. In the present study we have examined the influence of plasmid structure in relationship to gene expression in a peptide-mediated gene delivery system. The results not only establish important methodology that allow direct analysis of DNA stability by gel electrophoresis, but also report that peptide/DNA condensates are stable to sonicative shear stress and direct attack from serum endonucleases. These properties help to explain how peptide/DNA condensates are able to transform cells in culture in the presence of serum and suggest that a similar stability will be realized in vivo.

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Materials and Methods

CWK_8 (Alkylated Cys-Trp-Lys_8), CW[redacted]Alkylated Cys-Trp-Lys_{18}), and dimeric CWK_{18} (K_{18}WC-CWK_{18}) were synthesized and characterized as described previously.[19] pCMVL was produced in *E. coli* and purified using a Qiagen Ultrapure-100 kit (Santa Clarita, CA). TPCK-treated trypsin was obtained from Worthington Biochemicals (Freehold, NJ). Micro BCA protein assay reagent kit was obtained from Pierce (Rockford, IL). MEM, bovine calf serum, electrophoresis grade agarose, and LipofectAce (1:2.5 w/w dimethyldioctadecyammonium bromide and dioleolylphosphatidylethanolamine) were obtained from Gibco BRL (Gaithersburg, MD). Nru I restriction enzyme was purchased from Boehringer Mannhein (Indianapolis, IN).

Generation of Linear and Circular pCMVL—Supercoiled plasmid pCMVL[19] was linearized with Nru I which recognizes the sequence TCG/CGA at bp 206 of the pRC/CMV cloning vector from Invitrogen (San Diego, CA). One hundred units of Nru I was used to cleave 100 μg of pCMVL in 200 μL of SuRE/Cut buffer at 37 °C for 1 h. The linear DNA was purified by precipitation with 150 μL of ethanol at −20 °C followed by centrifugation at 13000 g for 5 min at 4 °C and then analyzed by 1% agarose gel electrophoresis.

Open circular pCMVL was prepared by creating single stranded nicks in the supercoiled pCMVL. The DNA (100 μg) was heated to 70 °C in 100 μL of TAE buffer, pH 8.0, for 1 h and then purified using ethanol precipitation and analyzed by gel electrophoresis.

Preparation and Characterization of Peptide/DNA Condensates—Peptide/DNA condensates were prepared at a DNA concentration of 50 μg/mL in 5 mM Hepes pH 7.4 using a stoichiometry of 0.3 nmol of peptide per μg of DNA. DNA (150 μL of 0.1 μg/μL) was added dropwise to a microfuge tube containing 4.5 nmol of peptide in 150 μL of buffer. Peptide/DNA condensates formed instantly, although physical measurements were carried out after 30 min to allow the particle size to stabilize.[19] Particle size analysis was performed on 350 μL of the undiluted DNA/peptide complex using a Nicomp 370 Autodilute Particle Sizer (Nicomp, Santa Barbara, CA).

Sonication of DNA Condensates—A 100 W Microson XL-2000 ultrasonic probe homogenizer (Kontes, Vineland, NJ) with a vibrational amplitude of 5 was used throughout the study. The probe tip was placed at 3/4 depth into a 1.5 mL microfuge tube containing 300 μL of sample. The sonication time was varied from 15 to 60 s, and the probe tip was washed between samples using deionized water.

Sonicated DNA samples were analyzed by gel electrophoresis on a 1% agarose gel prepared in TAE buffer (pH 8.0, 40 mM Tris acetate, 2 mM EDTA) containing 0.5 mg/mL ethidium bromide. Prior to electrophoresis, peptide/DNA condensates (2.5 μg DNA) were digested for 30 min with 5 μg of trypsin (0.2 units) prepared in a final volume of 50 μL of 5 mM Hepes pH 7.4 in order to release plasmid DNA from the condensate. The DNA (0.75 μg/15 μL) was combined with 3 μL of loading buffer (0.25 wt % bromophenol blue, 0.25 wt % xylene cyanol FF, and 30 wt % glycerol in water) and then loaded onto the gel and electrophoresed for 1.5 h at 70 V. DNA bands were visualized following destaining of the ethidium bromide on a transilluminator and photographed on Polaroid 667 black and white film.

DNA condensates were prepared using either CWK_8, CWK_{18} or dimeric-CWK_{18} and then adjusted to 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1 M sodium chloride and sonicated for 60 s to fragment uncondensed DNA. The samples were then digested with trypsin and electrophoresed on an agarose gel.

Serum Stability of Peptide/DNA Condensates—The DNAse activity in freshly prepared mouse serum was determined to be 4.2 units/μL according to the method of Kunitz.[20] CWK_{18}/DNA condensates (5 μg/100 μL) prepared in 5 mM Hepes pH 7.4 and 150 mM sodium chloride were combined with 100 μL of mouse serum and allowed to incubate at 37 °C for 3 h, while rapidly freezing 10 μL aliquots at intermediate time points. Prior to electrophoresis, 10 μL of 10 mg/mL SDS was added to each aliquot along with 5 μL of 68 mM EDTA and 3 μL of loading buffer, and samples were applied to an agarose gel containing 0.05 w/v% SDS and electrophoresed for 1 h at 70 V. The gel was destained in water for 24 h to remove SDS and increase the detection of the bands by ethidium bromide.

The stability of a LipofectAce/DNA complex was also studied by combining 30 μL of LipofectAce with 20 μL of 5 mM Hepes containing 150 mM sodium chloride and 50 μL of DNA (10 μg/100 μL of 5 mM Hepes pH 7.4). The complexes were then combined with 100 μL of mouse serum, incubated at 37 °C for 3 h while removing time points, and then electrophoresed as described above for peptide/DNA condensates.

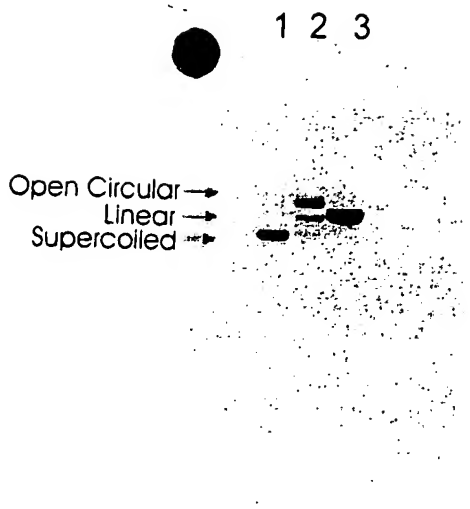

Figure 1—Gel electrophoresis of supercoiled, circular, and linear DNA. The result of converting supercoiled pCMVL (lane 1) to open circular (lane 2) and linear (lane 3) DNA is shown. Each lane was loaded with 0.5 μg of DNA and electrophoresed as described in Materials and Methods. The result of gene transfer with each type of DNA is described in Figure 2.

In Vitro Gene Transfer—Gene transfer experiments were performed on HepG2 cells grown to 40% confluence in MEM supplemented with 10% fetal bovine serum as reported previously.[19] The DNA condensate (10 μg of DNA) was applied to the cells in 2% fetal bovine serum with 80 μM chloroquine and allowed to incubate for 5 h after which time the media was replaced with MEM containing 10% fetal bovine serum.[19] After 24 h, cells were harvested and analyzed for the presence of luciferase. The expression level of luciferase was normalized for protein in each well, and the relative light units were converted to fmol of luciferase/mg of protein using a standard curve as reported previously.[19] Each experimental result represents the mean and standard deviation derived from a triplicate set of transfections.

Results

To establish a relationship between plasmid DNA structure and gene transfer efficiency, we first prepared circular DNA by base hydrolysis of supercoiled DNA. In its native form, plasmid DNA exists as a mixture of both supercoiled and open circular DNA forms that resolve on gel electrophoresis (Figure 1, lane 1). Treatment of plasmid DNA at pH 8.0 with elevated temperature (70 °C) accelerated the hydrolysis of supercoiled DNA to form predominantly circular DNA within 2 h (Figure 1A, lane 2). Linear DNA was prepared by restriction digestion with Nru I, which cleaved the plasmid prior to the CMV promoter, leaving the essential coding region for luciferase and the CMV promoter intact (Figure 1, lane 3).

Condensation of DNA with peptides possessing lysine repeats of either 18 or 36 residues resulted in the formation of fully condensed 50−70 nm diameter particles.[19] Linear, supercoiled, and circular DNA condensates could not be distinguished through particle size on QELS analysis (Table 1). However, their transfection efficiency was significantly different (Figure 2). The gene transfer efficiency of circular DNA was only reduced 10% compared to supercoiled DNA whereas linear DNA condensates were nearly 90% less efficient at transfecting cells and uncondensed DNA was inactive in mediating expression.

We then analyzed the influence of shear stress and endonuclease attack on the stability of supercoiled plasmid

Table 1—Particle Size Analysis of Peptide/DNA Condensates[a]

DNA morphology	particle population	
	diameter[b](nm)	σ[c] (nm)
supercoiled	46.9	32.4
circular	61.0	36.0
linear	72.6	37.4
sonicated[d]	44.8	28.8

[a] Peptide/DNA condensates were prepared at a concentration of 50 μg/mL of DNA and at stoichiometry of 0.3 nmol of CWK_{18} per μg of DNA. [b] Represents the mean diameter of particles. [c] Standard deviation of the population. [d] DNA sonicated for 60 s prior to condensation with CWK_{18}.

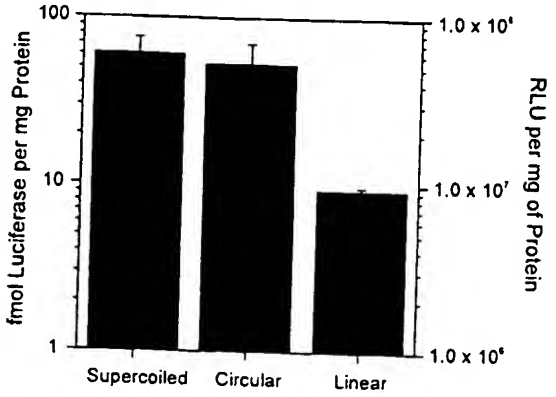

Figure 2—Gene transfer efficiency of supercoiled, circular, and linear DNA. The transfection efficiency of the three types of DNA shown in Figure 1 were compared. Each transfection utilized 10 μg of DNA combined with 3 nmol of CWK_{18} in 200 μL of 5 mM Hepes pH 7.4. The results establish a slight (10%) reduction in gene transfer when using open circular DNA and a 90% reduction when the DNA is linear relative to supercoiled DNA. Identical transfections with uncondensed DNA only resulted in 0.001 fmol of luceriferase per mg of protein.

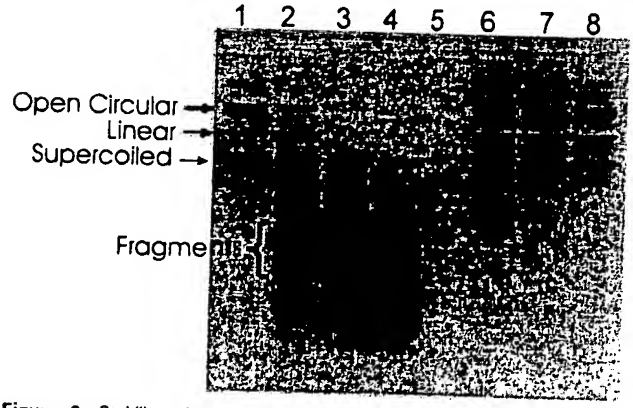

Figure 3—Stability of peptide/DNA condensates to ultrasonication. Gel electrophoresis with ethidium staining was used to demonstrate the fragmentation of plasmid DNA on sonication for 15, 30, and 60 s (lanes 2, 3, and 4) relative to standard DNA (lane 1). After complexation with CWK_{18} and sonication for 60 s, plasmid DNA failed to migrate on gel electrophoresis (lane 5). Treatment of the sonicated condensed DNA with trypsin restored migration but led to the formation of some linear DNA (lane 6). Omission of sonication (lane 7) or both sonication and condensation with CWK_{18} (lane 8) resulted in identical banding patterns indicating that linear DNA was an artifact of contaminated trypsin.

DNA. Condensed and uncondensed DNA were challenged with 100 W sonication for up to 60 s. Sonication of uncondensed plasmid DNA for as little as 15 s resulted in extensive fragmentation (Figure 3, lane 2), while prolonged sonication for 30–60 s further fragments DNA to form a finite size distribution of oligonucleotides as previously reported (Figure 3, lanes 3 and 4).[21]

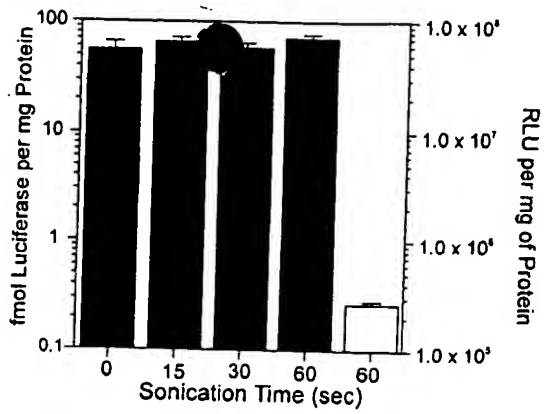

Figure 4—Gene transfer efficiency of sonicated peptide/DNA condensates. The expression of luciferase following in vitro transfection of HepG2 cells is compared for CWK_{18}/DNA condensates subjected to 0, 15, 30, and 60 s of sonication, relative to plasmid DNA sonicated for 60 s and then condensed with CWK_{18} (open bar).

In contrast, electrophoretic analysis of peptide condensed DNA sonicated for 60 s resulted in an empty lane (Figure 3, lane 5). This results from the failure of peptide/DNA condensates to dissociate and to stain with ethidium bromide. Trypsin was used to hydrolyze CWK_{18}, allowing the plasmid DNA to migrate and stain normally in the gel (Figure 3, lane 6). The formation of 10% linear DNA was the result of endonuclease contamination of trypsin. This was deduced from control experiments in which trypsin digestion of peptide/DNA condensates as well as uncondensed plasmid DNA both produced a linear DNA band (Figure 3, lane 7 and 8).

Peptide/DNA condensates were sonicated for up to 60 s and used to transfect HepG2 cells to establish that sonication also does not alter gene transfer efficiency. The results showed no change in gene transfer efficiency for sonicated DNA relative to unsonicated condensates (Figure 4). Fragmented DNA also formed small (45 nm) peptide/DNA condensates (Table 1), but these showed negligible gene expression activity (Figure 4).

Since the stability of peptide/DNA condensates may also be influenced by the solution ionic strength, we analyzed the dissociation of peptide/DNA condensates in the presence of increasing sodium chloride concentration in an attempt to disrupt the peptide/DNA binding. However, the failure to detect any DNA bands by gel electrophoresis after incubating the condensates with up to 5 M sodium chloride suggested that either the condensates failed to dissociate or they reformed during gel electrophoresis.

To distinguish between these alternatives, peptide/DNA condensates were treated with sodium chloride at concentrations ranging from 0 to 1 M and then sonicated for 60 s to fragment any uncondensed DNA. Trypsin was then added to hydrolyze CWK_{18} and allow the DNA to migrate into the gel during electrophoresis. The resulting gel established that peptide/DNA condensates dissociate at sodium chloride concentrations below 1 M as revealed by the presence of fragmented DNA (Figure 5).

The validity of the method was established by comparing the sodium chloride concentration required to dissociate three different peptides. Previously, we established that CWK_8 binds weakly to DNA but was able to fully condense DNA at a stoichiometry of 0.8 nmol per μg of DNA.[19] As observed in Figure 5A, the DNA remains protected by CWK_8 in 0.1 M sodium chloride (lane 2) but was degraded by sonication at all higher sodium chloride concentrations due to dissociation of the peptide/DNA complex. CWK_{18} possesses a significantly greater affinity for DNA resulting in complete condensation at 0.3 nmol per μg of DNA.[18] The

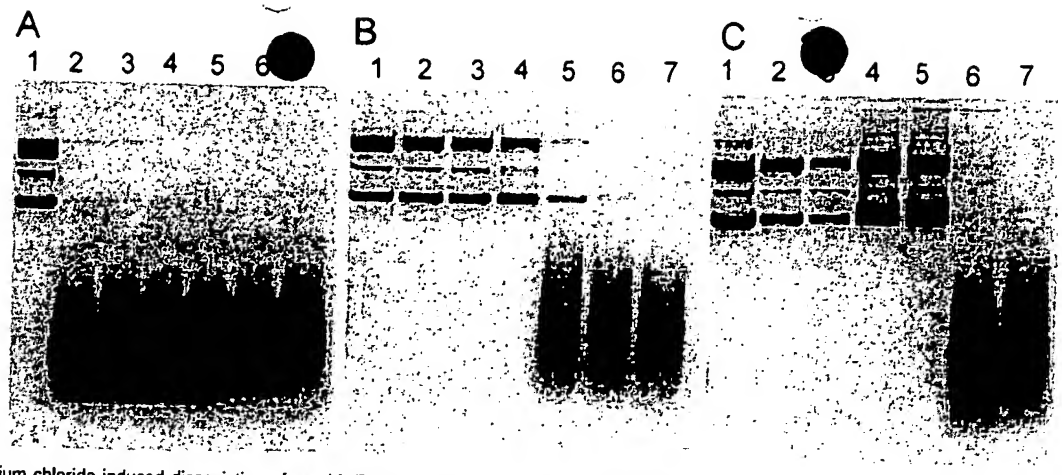

Figure 5—Sodium chloride induced dissociation of peptide/DNA condensates. The stability of peptide/DNA condensates to sonication in the presence of 0–1 M sodium chloride is demonstrated using gel electrophoresis. CWK_8 (panel A), CWK_{18} (panel B), and dimeric-CWK_{18} (panel C) DNA condensates were treated with 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1 M sodium chloride prior to 60 s sonication and trypsin digestion. The dissociation of the peptide/DNA condensate was observed at above 0.1 M for CWK_8 (panel A), above 0.4 M CWK_{18} (panel B), and above 0.6 M for dimeric-CWK_{18} (panel C) DNA condensates.

results shown in Figure 5B support this by demonstrating protection of the DNA by CWK_{18} at concentrations up to 0.4 M sodium chloride. Likewise, dimeric-CWK_{18} was previously found to have a slightly higher affinity for DNA but produced condensates that were the same size as CWK_{18}.[19] In agreement with these results, dimeric-CWK_{18}/DNA condensates were more stable as demonstrated by their ability to resist sonicative-induced fragmentation up to 0.6 M sodium chloride.

To study the serum stability of peptide/DNA condensates required modification of the gel electrophoresis approach. In the presence of serum, trypsin was incapable of completely hydrolyzing the peptide to release DNA, resulting in bands that migrated slower than open circular DNA on gel electrophoresis (data not shown). Consequently, SDS was found to be an effective agent to dissociate the peptide/DNA complexes present in serum. The optimal result was obtained by adding 0.05 w/v% of SDS in the agarose gel and running buffer along with 0.3 w/v% SDS in the loading lane.

Incubation of either DNA, peptide/DNA condensates, or LipofectAce/DNA complexes with freshly prepared mouse serum adjusted to 0.15 M sodium chloride was used to establish the in vitro stability of DNA. Direct analysis of the incubation time points by SDS-agarose electrophoresis established that uncondensed DNA rapidly converted from supercoiled to circular DNA and then began to form linear DNA within a 5 min incubation period (Figure 6A, lane 2). Further incubation resulted in progressive formation of linear DNA which then degraded to smaller oligonucleotides within 1 h and resulted in complete fragmentation of DNA within 3 h (Figure 6A, lanes 6–8). Conversely, CWK_{18} condensed DNA was stable when exposed to serum during a 3 h incubation period (Figure 6B), demonstrating the complete preservation of supercoiled DNA.

This result suggested that the dissociation of CWK_8/DNA condensates in normal saline (0.15 M) could leave DNA exposed to serum endonucleases. Incubation of CWK_8/DNA condensates with serum resulted in a degradation profile that was identical to that observed for uncondensed DNA (Figure 6C). This adequately explains the three-orders of magnitude difference in the transfection efficiency previously observed when transfecting cells with CWK_{18} and CWK_8/DNA condensates in the presence of fetal calf serum.[19]

This same rational may also explain the ineffectiveness of LipofectAce/DNA transfection in the presence of serum. To establish this point, LipofectAce/DNA complexes were incubated in serum, and time points ranging from 0 to 3 h were analyzed by gel electrophoresis (Figure 6D). Although some protective effect was conferred by the presence of the cationic lipid formulation, the DNA still underwent a complete conversion from supercoiled to circular and linear DNA within 1 h and was 50% depolymerized during the 3 h incubation.

Discussion

The stability of a DNA formulation in serum is fundamental to its successful application in vivo since premature metabolism results in the generation of fragmented DNA which lacks gene transfer potency. The utility of gel electrophoresis to study the stability of plasmid DNA complexed with peptides or lipids in a gene delivery formulation has been limited because of the interference imparted by the these biopolymers which retard or distort the electrophoretic migration of DNA. The present results establish that the addition of SDS to agarose gel electrophoresis or, the use of trypsin to remove peptide, allow direct analysis of the status of DNA in a complex formulation and in serum. The use of radiolabeled DNA will further advance these studies by allowing a quantitative analysis of DNA recovery.

In an effort to understand how the serum stability of peptide/DNA condensates correlates with gene expression, we first analyzed how changes in plasmid structure influence gene transfer efficiency. The results presented in Figures 1 and 2 demonstrate that the conversion of DNA from supercoiled to circular only has a minor effect on the gene transfer efficiency. However, subsequent conversion to linear DNA reduces gene expression by 90% whereas transformation to oligonucleotide fragments via sonication reduced gene expression nearly 1000-fold (Figure 2).

We were intrigued to find that high-intensity sonication of CWK_{18}/DNA condensates for up to 60 s failed to degrade the DNA (Figure 3). It is also interesting that sonicatively fragmented DNA was still able to bind to CWK_{18} and produce condensates that were indistinguishable in size relative to those prepared from supercoiled, circular, or linear DNA (Table 1). Thus, it is evident that the poor transfection performance of fragmented DNA is not because of the failure to form condensate but rather directly related to the stability of the DNA.

We cannot explain the mechanism by which DNA folds to produce particles of a defined size, but it must be

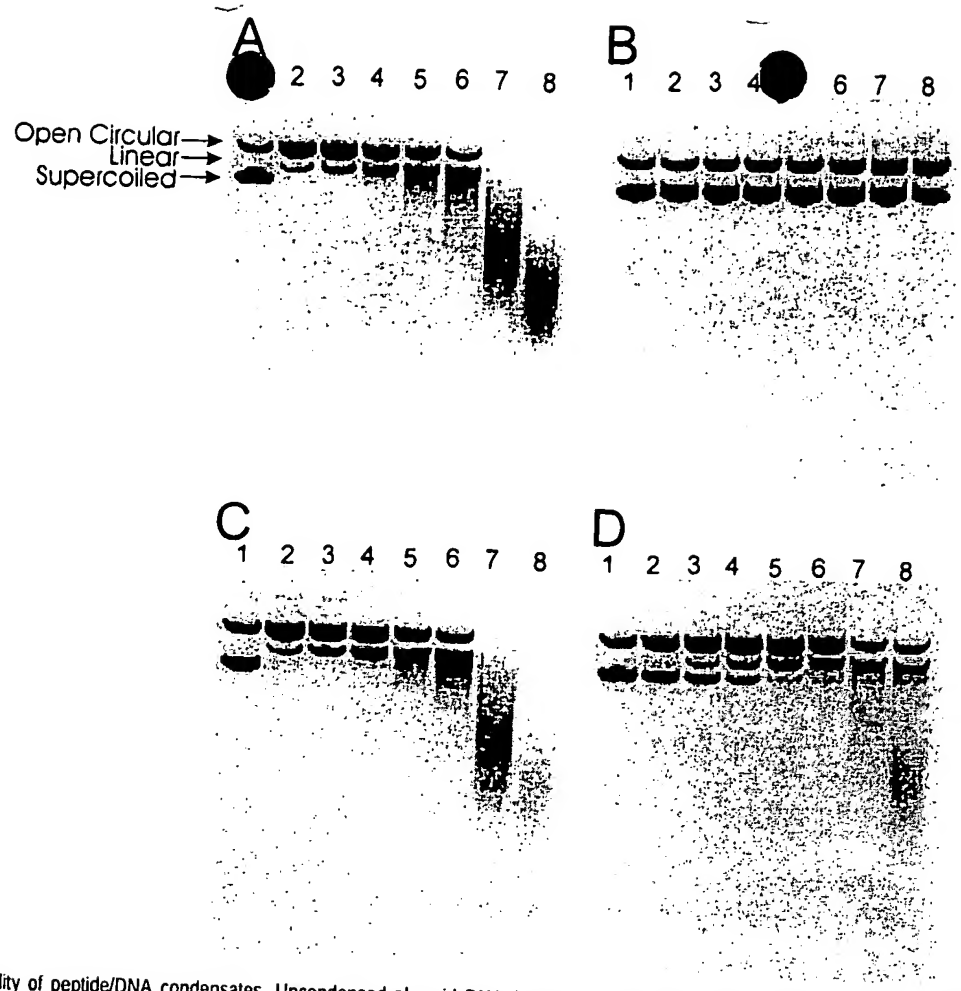

Figure 6—Serum stability of peptide/DNA condensates. Uncondensed plasmid DNA (panel A), CWK_{18}/DNA condensates (panel B), CWK_8/DNA condensates (panel C), and LipofectAce/DNA complexes (panel D) were incubated with mouse serum at 37 °C and analyzed by gel electrophoresis as described in Materials and Methods. Time points were analyzed at 0, 5, 15, 30, 45, 60, 120, and 180 min in lanes 1–8, respectively. The rapid conversion of supercoiled DNA to linear DNA is evident in panels A and C, and the formation of fragmented DNA is nearly complete at 2 h indicating a lack of protection afforded by CWK_8. LipofectAce/DNA complex showed a similar profile but the DNA fragmentation was slower than for uncondensed DNA. Panel B illustrates the protective effects of CWK_{18} during the same 3 h incubation.

intrinsically related to the nature of peptide rather than the size or polydispersity of the DNA since it is clear that peptide size influences the properties of condensates.[19,22] A practical outcome of this analysis of peptide/DNA stability is that high power sonication could be used as a means to reduce particle size while still preserving DNA structure when developing complex formulations for gene therapy that require shear stress, such as liposomes[18] or aerosols.[8]

The dissociation of peptide/DNA complexes in sodium chloride allowed for the development of an assay to define the condensation status of DNA in a complex solution. The data presented in Figure 5 clearly demonstrate that the dissociation of peptide from DNA at a defined salt concentration depends on the affinity of different peptides binding to DNA. The data not only established that CWK_{18} retains its ability to bind to DNA in 0.4 M sodium chloride but also revealed that CWK_8 dissociates at a concentration of 0.1 M sodium chloride. These results predicted that CWK_{18} would protect DNA from endonucleases in normal saline (0.15 M sodium chloride) whereas CWK_8 would not protect DNA. This proved to be the case, in that there was no distinction between DNA serum stability in the presence or absence of CWK_8 whereas the complete protection of DNA was observed when complexed with CWK_{18} (Figure 6).

An examination of the serum stability of LipofectAce/DNA complexes determined that the DNA was more stable than uncondensed plasmid DNA but still degraded to oligonucleotides within a couple of hours. These results adequately explain the lack of potency when transforming cells with uncondensed DNA and when using LipofectAce in the presence of serum. Moreover, the data suggest a possible explanation for the lack of correlation between in vitro and in vivo gene transfer results for certain gene delivery vehicles.

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